Ana Carina Martins Pereira

ROLE OF POLYMORPHISMS IN PROSTAGLANDIN E2 (PGE_2) PATHWAY GENES IN COLORECTAL CARCINOGENESIS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Prof. Doutor Mário Jorge Dinis Ribeiro

Categoria – Professor Associado Convidado com Agregação

Afiliação – Faculdade de Medicina da Universidade do Porto.

Coorientador – Prof. Doutor Rui Manuel de Medeiros Melo Silva

Categoria – Professor Associado Convidado com Agregação

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka!' but 'That's funny...'

Isaac Asimov

To my parents…

…my inspiration, my strength.

AGRADECIMENTOS

Ao Prof. Doutor **Mário Dinis-Ribeiro**, orientador desta tese, por todo o apoio, motivação e confiança depositada, que se revelaram imprescindíveis para a conclusão da mesma. Pela paciência e pelo conhecimento transmitido durante este percurso. Obrigada por acreditar em mim.

Ao Prof. Doutor **Rui Medeiros**, co-orientador desta tese, pela paciência, compreensão e palavras de motivação. Obrigada por confiar no meu trabalho.

Núcleo Regional do Norte da Liga Portuguesa Contra o Cancro, em particular ao **Dr. Vítor Veloso**, por me concederem a bolsa que permitiu a conclusão desta tese.

Ao Prof. Doutor **Paul Farrell**, Coordenador da *Virology Section of the Department of Medicine from the Imperial College London* por prontamente me receber de forma desinteressada no seu laboratório e entusiasticamente me orientar nos trabalhos *in vitro*. Ao Dr. **Claudio Elguetta-Karstegl**, pela orientação prática e restantes elementos do grupo por me fazerem sentir em casa.

Ao **Dr. Luís Moreira-Dias**, diretor do Serviço de Gastrenterologia do Instituto Português de Oncologia do Porto (IPO-Porto), por ter facilitado a realização deste estudo e o contacto com o doente oncológico.

Ao Prof. Doutor **Pedro Pimentel-Nunes** e Dra. **Catarina Brandão**, do Serviço de Gastrenterologia IPO-Porto, e Prof. Doutor **Ricardo Marcos-Pinto**, do Serviço de Gastrenterologia do Centro Hospitalar do Porto (CHP) pela contribuição fundamental na seleção e recrutamento de doentes com tumores colo-retais e obtenção de amostras biológicas.

A todos os médicos, enfermeiras e auxiliares de ação médica do Serviço de Gastrenterologia e Clinica de Patologia Digestiva do IPO-Porto, nomeadamente à Enf. Chefe **Fátima Teixeira** pelo auxílio prestado no contacto com os doentes e à Dra. **Cristina Moreira** pelo imprescindível apoio e horas dedicadas na fase inicial deste projeto.

AGRADECIMENTOS

A todos os **doentes** que aceitaram participar neste estudo pelo contributo que deram.

Ao Prof. Doutor **Rui Henrique**, diretor do Serviço de Anatomia Patológica do IPO-Porto, por colaborar neste projeto e facilitar o acesso às amostras biológica essenciais para este projeto.

À Dra. **Ana Galaghar**, do Serviço de Anatomia Patológica do IPO-Porto pela pronta colaboração e muitas horas dedicadas à seleção e revisão dos cortes histológicos e à técnica **Fernanda Silva** pelo apoio prático no corte dos blocos histológicos.

À Dra. **Paula Magalhães**, do Serviço de Genotipagem e Cultura Celular do Instituto de Biologia Molecular e Celular do Porto (IBMC) pelos preciosos ensinamentos na área da cultura celular.

Ao **Grupo de Oncologia Molecular** do IPO-Porto, em especial ao **Hugo** e às **Joanas,** mais do que colegas de trabalho, amigos, pelos desabafos, gargalhadas e tertúlias que em muito contribuíram para a condução dos trabalhos. Obrigada **Joana** e **Marlene** pela vossa dedicação na reta final deste projeto. Ao **Ricardo,** investigador entusiasta, por todas as conversas inspiradoras ao longo destes anos.

À **Sara** e à **Carla**, estudantes de mestrado, pelo entusiástico e preponderante apoio prestado no desenvolvimento dos trabalhos.

Aos **Meus Amigos** que incansavelmente ouvem os meus desabafos, que festejam as minhas conquistas, mas principalmente pelo encorajamento e motivação, que foram essenciais durante este processo. Ao **Luís,** amigo sempre presente, pelos conselhos práticos e palavras de encorajamento.

Aos **Meus Pais**, pelo inesgotável apoio e dedicação. Por me ensinarem que mesmo nos momentos menos fáceis temos de lutar pelas nossas convicções e objetivos. Obrigada!!!

APOIO FINANCEIRO

Este projeto foi desenvolvido no Grupo de Oncologia Molecular do Centro de Investigação do Instituto Português de Oncologia do Porto (IPO-Porto) em colaboração com os Serviços de Gastrenterologia do IPO-Porto e Centro Hospitalar do Porto e foi possível com o apoio financeiro descriminado de seguida:

- Bolsa individual de investigação atribuída pela Liga Portuguesa Contra o Cancro – Núcleo regional do Norte – 2014

  Role of polymorphisms in prostaglandin E2 (PGE2) pathway genes in colorectal carcinogenesis

- Bolsa de Investigação atribuída pelo Centro de Investigação do IPOP – 2012

  Polymorphisms in PGE2 pathway genes (COX-2/HPGD/ABCC4/SLCO2A1) and colorectal carcinogenesis: definition of a risk model for early screening and chemoprevention

- Bolsa individual de investigação co-financiada pelo Programa Operacional Potencial Humano (POPH)/ Fundo Social Europeu (FSE) e Fundação para a Ciência e Tecnologia - FCT (SFRH / BD / 64805 / 2009) – 2009

  Polymorphisms in PGE-2 metabolizing genes and colorectal carcinogenesis: definition of a risk model for early screening and Chemoprevention

- Bolsa de investigação atribuída pela Sociedade Portuguesa de Gastrenterologia (SPG) – 2007

  Caracterização farmacogenómica da COX na carcinogénese do cólon

UNIÃO EUROPEIA
Fundo Social Europeu

TABLE OF CONTENTS

TABLES OF CONTENTS

LIST OF ABREVIATIONS & SYNONYMS

A	Adenine
AA	Arachidonic acid
ABCC4	ATP-binding cassette sub-family c member 4
APC	Adenomatous polyposis coli
BMI	Body mass index
C	Cytosine
CI	Confidence interval
COX	Cyclooxygenase
CRC	Colorectal cancer
Ct	Cycle threshold
CVC	Cross-validation consistency
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FFPE	Formalin-fixed paraffin embedded
FOBT	Feacal occult blood test
FPRP	False positive report probability
G	Guanine
GI	Gastrointestinal
HWE	Hardy-Weinberg equilibrium
HR	Hazard ratio
KRT20	Cytokeratine 20
LD	Linkage desiquilibrium
MDR	Multifactor dimensionality reduction
MRP4	Multidrug resistance-associated protein 4
NSAID	Nonsteroidal anti-inflamatory drugs
OR	*Odds ratio*
PCR	Polymerase chain reaction
PG	Prostaglandin
PGT	Prostaglandin transporter
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SD	Standart deviation
SLCO2A1	solute carrier organic anion transporter family, member 2A1
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
T	Thymine
TF	Transcription factors
UPY	Unit packs years
UTR	Untranslated region
15-PGDH	15-hydroxyprostaglandin dehydrogenase

Some discrepancy in polymorphisms nomenclature can be noticed accross the chapters. The correspondence is as follows:

Chapter IA	Chapter IB and III	Chapters IV to VI
-1329A>G	-1195A>G	rs648966
-899G>C	-765G>C	rs20417
*849T>C	8473T>C	rs5275

OUTLINE OF THE THESIS

This thesis is structured in **eight** major chapters:

In **chapter I**, the background for this study will be presented, providing a clinical contextualization and stressing the role of four key proteins (COX-2/15-PGDH/MRP4/PGT) involved in prostaglandin E_2 (PGE_2) regulation in tumor development and the potential contribution of polymorphisms for colorectal cancer burden. This chapter is further subdivided in **A** and **B**.
A systematic review and meta-analysis on published studies addressing the contribution of *COX* polymorphisms in gastrointestinal carcinogenesis, including the description of all *COX-2* polymorphisms previously analyzed in colorectal tumors will be provided to readers in **Chapter IA.**
In **Chapter IB**, a case-control study will be described and discussed to demonstrate as proof-of-concept, the impact of three specific *COX-2* polymorphisms, shown to modulate the susceptibility for colorectal tumors in the meta-analysis, in the development of CRC in a Northern Portuguese population.

The purpose and aims of this thesis are disclosed in **Chapter II**.

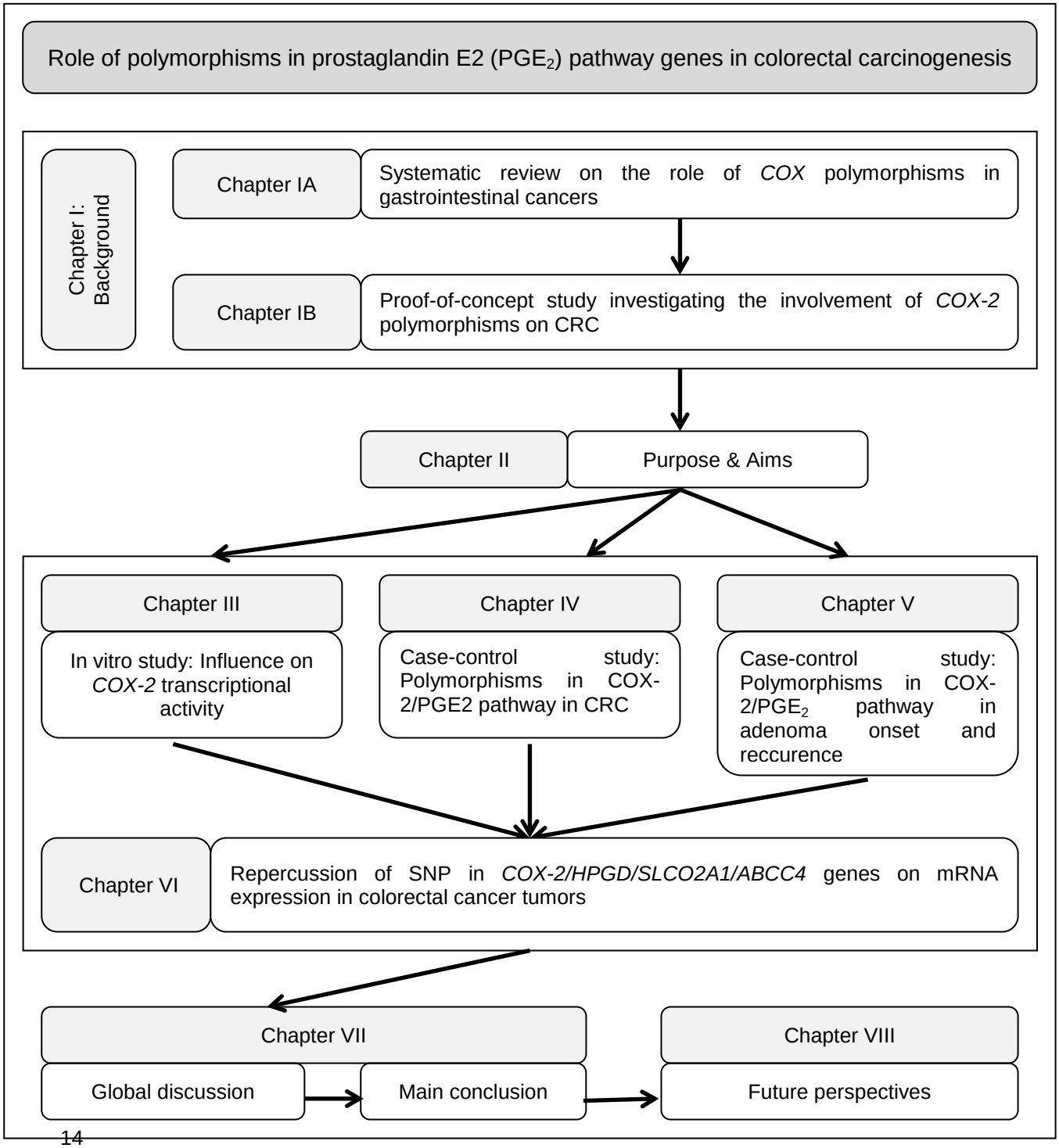
Considering that an increased risk for CRC in individuals carrying of rs689466G allele (-1195G allele) in *COX-2* gene was reported in the proof-of-concept study and the lack of consistency noticed among published studies, in **Chapter III**, an *in vitro* approach assessing the repercussion of this SNP in *COX-2* transcriptional activity in CRC cell lines is displayed, offering a biological plausibility behind the epidemiological data.

Chapters IV and **V** are characterized by two retrospective observational studies addressing the influence of *COX-2* and three other important genes regulating PGE_2 leveles in CRC (*HPGD, SLCO2A1* and *ABCC4),* on the predisposition for CRC and colorectal precancerous lesions onset, respectively. Furthermore, **Chapter V** also reports not only the influence on the development of metachronous adenomas in patients with previous history of adenomas, but also the time at which those lesions recur.

OUTLINE OF THE THESIS

In **Chapter VI**, a study assessing the repercussion of SNPs identified as risk markers for colorectal carcinogenesis on **Chapters IV** and **V** on mRNA expression is reported.

Finally, in **Chapter VII** a global discussion and main conclusion will be provided, followed by the suggestion for future research in this field of knowledge presentedin **Chapter VIII**.



LIST OF PUBLICATIONS

The list of publication that integrated the experimental component of this thesis is hereby presented:

I. Pereira C, Sousa H, Silva J, Brandão C, Elgueta-Karstegl C, Farrell PJ, Medeiros R, Dinis-Ribeiro M. *The -1195G allele increases the transcriptional activity of cyclooxygenase-2 gene (COX-2) in colon cancer cell lines.* Mol Carcinog 2014;53 Suppl 1:E92-5. doi: 10.1002/mc.22049. Epub 2013 Jun 18.

Jounal impact factor: 4.27

Oral presentation in the plenary section at *Semana Digestiva* 2012, Porto, Portugal

Poster presentation at 20th United European Gastroenterology Week (UEGW), Amsterdam, Holand, 2012

II. Pereira C, Queirós S, Galaghar A, Sousa H, Pimentel-Nunes P, Brandão C, Moreira-Dias L, Medeiros R, Dinis-Ribeiro M. *Genetic variability in key genes in prostaglandin E_2 pathway (COX-2/HPGD/ABCC4/SLCO2A1) and their involvement in colorectal cancer development.* PLoS One 2014 Apr 2;9(4):e92000. doi: 10.1371/journal.pone.0092000. eCollection 2014. Jounal impact factor: 3.73

Poster presentation at 20th UEGW, Amsterdam, Holand, 2012. Distinguished has a Poster of Excellence

III. Pereira C, Queirós S, Galaghar A, Sousa H, Marcos-Pinto R, Pimentel-Nunes P, Brandão C, Moreira-Dias L, Medeiros R, Dinis-Ribeiro M. *Polymorphisms in prostaglandin E_2 (PGE_2) pathway genes alter the risk for colorectal adenoma recurrence after polypectomy: a chance for individualized surveillance?* (Submitted for publication).

LIST OF PUBLICATIONS

IV. Pereira C, Ribeiro J, Queirós S, Lima L, Sousa H, Galaghar A, Pimentel-Nunes P, Brandão C, Moreira-Dias L, Medeiros R, Dinis-Ribeiro M. *Influence of genetic polymorphisms in prostaglandin E_2 (PGE_2) pathway on mRNA expression of COX-2, HPGD, SLCO2A1 and ABCC4 genes in colorectal tumors.* (Submitted for publication)

Furthermore, two additional articles were included in the background chapter that were fundamental for the design of this thesis:

- Pereira C, Medeiros RM, Dinis-Ribeiro MJ. *Cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis: are conclusive results available?* Eur J Gastroenterol Hepatol 2009;21(1):76-91. doi: 10.1097/MEG.0b013e32830ce7ba.

  Journal impact factor: 1.92

- Pereira C, Pimentel-Nunes P, Brandão C, Moreira-Dias L, Medeiros R, Dinis-Ribeiro M. *COX-2 polymorphisms and colorectal cancer risk: a strategy for chemoprevention*. Eur J Gastroenterol Hepatol 2010;22(5):607-13. doi: 10.1097/MEG.0b013e3283352cbb.

  Journal impact factor: 1.92

  Oral presentation in the plenary section at the *XXIX Congresso Nacional de Gastrenterologia e Endoscopia Digestive*, Porto, Portugal, 2009.

  Distinguished with the best oral communication award.

  Oral presentation at the GASTRO 2009 – UEGW/WCOG, London, England, 2009.

  Awarded with a travel grant -100 best submitted Basic science abstract.

SUMMARY

Colorectal adenomatous polyps are well-characterized CRC precursors upon which the majority of CRC will develop in 10-15 years. Although the implementation of population-based CRC screening guidelines focused on the endoscopic detection and removal of these precancerous lesions is highly recommended, the compliance rates are far from the desirable for a sussessfull impact on CRC burden.

Complementary, aspirin regular has been consistently effective in the primary prevention of colorectal tumors, by targetting the cyclooxygenase-2 (COX-2) enzyme, nevertheless its use is currently hampered by the onset of serious gastrointestinal side effects in average-risk population. So, the challenge for CRC prevention falls in the identification of biomarkers that could target higher-risk populations for colorectal screening and/or chemopreventive strategies.

COX-2-derived prostaglandin E_2 (PGE_2), the major PG produced in colorectal tumors, plays a key contribution to the hallmarks of cancer, by stimulating cell proliferation, invasiveness and migration, enhancing angiogenesis, evading apoptosis and modulating the antitumor immune response.

In a preliminar study involving 373 participants from the Northern region of Portugal, we reported the involvement of a specific polymorphism in *COX-2* gene on the susceptibility for CRC development. In opposition, with earlier reports, individuals carrying the rs689466G allele (-1195A>G) were not only at an increased susceptibility but also had a 7-years antecipation on CRC development.

With this in mind, and to lend further support to the epidemiologic data, we carried-out a functional study and observed that *COX-2* promoters' containing the rs689466G allele had a two to three-fold increase in transcriptional activity in comparison with the ones encompassing the ra689466A allele in CRC cell lines.

The positive finding in the proof-of-concept study, suggested we were focusing on the right genetic pathway, so we decided not only to increase our study population but also expand our search for genetic biomarkers to other key players in COX-2/PGE_2 pathway. The *hydroxyprostaglandin dehydrogenase* (*HPGD*) gene directly counteracts the COX-2 oncogenic PGE_2 pathway. The *ATP-binding cassete sub-family C member 4* (*ABCC4*) gene and *solute carrier organic anion transporter*

SUMMARY

family, member 2A1 (*SLCO2A1*) gene, code for specific prostaglandin membrane transporters that regulate PGE_2 levels in the extracellular microenvironment.

Seven tagSNPs were implicated in CRC development using a tagSNP approach including 51 genetic polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* PGE_2 pathway and nearly 750 participants: rs689466, rs1346271, rs1426945, rs6439448, rs7616492, rs1751051 and rs1751031. Consistently, individuals ever-smokers carriers of rs689466 GG homozygous genotype had a nearly six-fold increased susceptibility for CRC onset (95%CI: 1.49-22.42, *P*=0.011). Furthermore, the multifactor dimensionality reduction (MDR) analysis identified an overall four-factor best gene-gene interactive model, including the rs1426945, rs6439448, rs1751051 and rs1751031 polymorphisms (cross-validation consistency: 10/10, accuracy: 0.6957; OR=5; 95%CI: 3.89-7.02, *P*<0.001).

We then questioned if polymorphisms in the aforeentioned genes would also be relevant in early stages of colorectal tumor development and recurrence of colorectal adenomas.

Ten tagSNPs were identified as susceptibility biomarkers for the development of colorectal adenomas: the rs689466 in *COX-2*, the rs2555639, rs1346271, rs1863642 and rs12500316 in *HPGD*, the rs6439448 and rs1131598 in *SLCO2A1* and rs9524821, rs1751051 and rs1678405 in *ABCC4* genes. The haplotype carying the rs9524821 and rs1751051 SNPs in *ABCC4* gene had a risk of 3.9 for adenomas development (95%CI:2.28-6.65, *P*<0.001). Furthermore, the best four-locus gene-gene interaction model included the rs1346271, rs186342 and rs12500316 SNPs in *HPGD* and rs1678405 in *ABCC4* genes and was associated with a 13-fold increased susceptibility (95%CI:3.84-46.3, *P*<0.001, cross-validation (CV) accuracy: 0.78 and CV consistency: 8/10*)*. Interesting, in high risk patients the rs1678405 *ABCC4* SNP had a lower HR and half the crude risk for adenoma recurrence at 36 months, when comparing with the overall high risk patients (7% vs 14%).

SUMMARY

A functional study characterizing the influence of polymorphisms previously identified as risk markers on the genes' mRNA expression was performed in colonic mucosa. This approach provided the biological plausibility behind some of the associations lending further surport to the involvement of genetic variants in PGE_2 pathway in colorectal carcinogenesis. In fact, the rs689466GG genotype, reported to have a higher transcriptional activity in CRC cell lines was further associated with a seven-fold *COX-2* overexpression in CRC tissues (-1.57±0.10 vs -4.42±1.58 with the AA genotype, *P*=0.021).

Although additional studies are needed, specific low penetrance genes in the pro-carcinogenic PGE_2 pathway appear to modulate the genetic susceptibility for colorectal tumors onset and recurrence. A clearer understanding on CRC etiology through the identification of biomarkers of colorectal carcinogenesis might allow a better definition of risk models that are more likely to benefit from targeted preventive and surveillance strategies to reduce CRC burden.

SUMÁRIO

Pólipos adenomatosos são lesões premalignas a partir dos quais a maioria dos cancros colo-retais (CCR) se irão desenvolver em média após 10-15 anos. Embora a implementação de rastreio de CCR através da detecção e remoção endoscópica dessas lesões seja recomendado a aderência a esses programas estão longe do desejável para um notório impacto na redução de CCR.

O consumo regular da aspirina tem sido consistentemente eficaz na prevenção primária de tumores colo-rectal por inibir a enzima cyclooxygenase-2 (COX- 2), no entanto, o seu uso está actualmente comprometido pelo desenvolvimento de efeitos secundários graves a nível gastrointestinal em população com risco médio para CCR. Assim, o desafio na prevenção de CCR passa pela identificação de biomarcadores que permitam direcionar populações com um risco aumentado para estratégias otimizadas de rastreio e prevenção primária de CCR.

A prostaglandina E_2 (PGE_2) produzida pela COX-2, desempenha um papel importante na activação de várias vias carcinogénicas, incluindo estimulação da proliferação celular, invasão, migração celular, inibição da apoptose e imunossupressão.

Num estudo preliminar, envolvendo 373 participantes da região Norte de Portugal, um polimorfismo especifico no gene *COX-2* foi implicado no desenvolvimento de CCR. Contrastando com a bibilografia disponível, indivíduos portadores do alelo rs689466G (-1195G), apresentaram não só um aumento na susceptibilidade, assim como, uma antecipação no diagnóstico de CCR.

Tendo este trabalho como base desenvolvemos um estudo funcional que permitiu constatar que a região promotora contendo o alelo rs689466G conduz um aumento 2 a 3 vezes superior na actividade transcripcional do gene *COX-2*.

Posteriormente, decidimos não só aumentar o número de participantes no nosso estudo caso-contolo, como expandir a pesquisa da variantes genéticas a outros genes relevantes na via da COX-2/PGE_2.

O gene *hydroxyprostaglandin desidrogenase* (HPGD) antagoniza os efeitos carcinogénicos da via PGE_2. O gene *ATP-binding cassete member sub- family C 4 (ABCC4)* e o *solute carrier organic anion transporter family, member 2A1* (*SLCO2A1*), que codificam transportadores de membrana específicos para o

transporte de prostaglandina, regulam os níveis de PGE_2 no microambiente extracelular.

Sete *tagSNP*s foram associados ao desenvolvimento de CCR, numa análise que incluiu 51 polimorfismos nos genes *COX-2/HPGD/SLCO2A1/ABCC4* em cerca de 750 participantes: rs689466, rs1346271, rs1426945, rs6439448, rs7616492, rs1751051 e rs1751031. Consistentemente, indivíduos ex ou atuais fumadores portadores do genótipo homozigótico rs689466GG apresentaram um aumento na susceptibilidade em 6 vezes (IC95%:1.49-22.42, *P*=0.011). Além disso, observou-se uma interação entre quatro polimorfismos (rs1426945, rs6439448, rs1751051 e rs1751031) que conduziu a um aumento no risco cinco vezes superior para CCR (IC95%:3.89-7.02, *P* <0.001).

Em seguida, questionamos se polimorfismos nos genes mencionados anteriormente também seriam relevantes em fases iniciais de desenvolvimento de CCR e recorrência de adenomas colo-rectais.

Dez tagSNPs foram identificados como biomarcadores de susceptibilidade para o desenvolvimento de adenomas: o rs689466, rs2555639, rs1346271, rs1863642, rs12500316, rs6439448, rs1131598, rs9524821, rs1751051 e o rs1678405. O haplótipo portador dos polimorfismos rs9524821 e rs1751051 no gene *ABCC4* apresentaram um risco para o desenvolvimento de adenomas de 3.9 (IC95%: 2.28–6.65, *P*<0.001). Curiosamente, em pacientes de alto risco o polimorfismo rs1678405 no gene *ABCC4* apresentou um HR inferior a metade do risco bruto de recorrência de adenoma aos 36 meses, comparativamente com os pacientes de alto risco (7% vs 14%).

De forma a caracterizar funcionalmente os polimorfismos anteriormente identificados como marcadores de risco, foi avaliada a repercussão destes na expressão génica. Esta abordagem suportou o envolvimento de variantes genéticas em genes da via da PGE_2 na carcinogénese colorectal. De fato, o genótipo rs689466GG que conduz a um aumento na actividade transcripcional associou-se a uma sobre-expressão de *COX-2* em tecidos de CCR (-1.57±0.10 vs -4.42±1.58 parao genótipo AA, *P*=0.021).

SUMÁRIO

Embora sejam necessários mais estudos, polimorfismos na via pró-carcinogénica PGE_2 parecem modular a suscetibilidade genética para o desenvolvimento e recorrência de tumores colo-retais. Uma melhor compreensão sobre a etiologia de CCR através da definição de biomarcadores de suscetibilidade colo-retal pode permitir uma melhor definição de modelos de risco, que permitirão otimizar as atuais estratégias de prevenção, rastreio e vigilância de tumores colo-rectais.

CHAPTER I: BACKGROUND

1.1. Colorectal cancer

1.1.1. *Epidemiology and risk factors*

Colorectal cancer (CRC) is malignant neoplasm arising from the lining of large intestine and represents a major public health problem worldwide, with an anual incidence of approximately 1.36 million cases and a mortality of nearly 693.881. Geographically, there is at least a ten-fold variation in CRC burden among both genders, with the highest rates reported in the more developed regions (65.8 per 100.000 in males), as can be observed in Figure 1 [1].

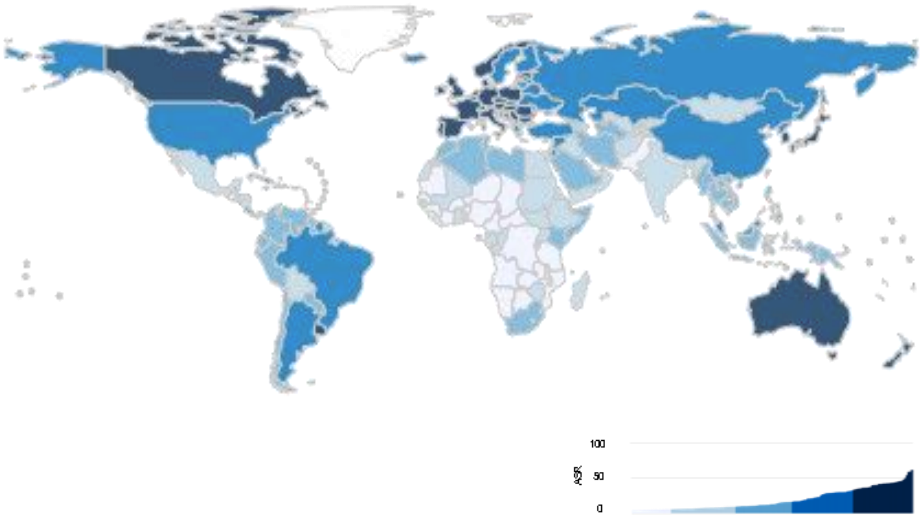


Figure 1. Incidence distribution of colorectal cancer among males in 2012 (per 100.000). Retrieved from Globocan 2012 online database (http://globocan.iarc.fr/).

In European countries, CRC is the third most common cancer in males (241.813 cases, 13% of total) and the second in females (205.323, 13% of cases). In 2012, 214.814 deaths were estimated due to CRC, making it the second most frequent cause of cancer death (12% of mortality) [1].

Unfortunately, the epidemiological picture in Portugal is even worse. CRC is the second leading neoplasia with the highest incidence, only behind prostate and breast cancers in males (4209, 15% of cases) and females (2920, 14% of total), respectively. Overall 90% of all cases are diagnosed in individuals with or over 60 years of age and males have a 44% higher incidence, with a cumulative risk for the development of CRC of 5% in contrast with the 3% reported in females [1].

The burden of CRC is expected to increase over the next decades in developed countries as a reflection of population aging and growth, through a complex interaction between inherited susceptibility and environmental factors [1-5]

Approximately 75% of patients have neither a clear family history nor any know predisposition condition [6]. In this context, the increasingly adoption of a “westernized” lifestyle, including physical inactivity, obesity, smoking, high consumption of red or processed meats and moderate-to-heavy alcohol drinking is believed to be a major determinant in the occurrence of sporadic cancer [4,5,7-9]. In fact, Kirkegaard and colleagues [10], suggest that following a healthy lifestyle could reduce the occurrence of CRC by 25%.

Individuals with first-degree relatives diagnosed with CRC, particularly under the age of 45 years, have a 2 to 3-fold increased risk for developing this neoplasia [11]. Furthermore, personal histories of CRC, chronic inflammatory bowel diseases (IBD), or previous occurrence of colorectal adenomas, known precancerous lesions, are also associated with CRC development [12,13]. The latter will be further explored in the next session.

Five-percent of cases are associated with one of two well-defined genetic syndromes that are characterized by the development of CRC at an early age [14,15]. The familial adenomatous polyposis (FAP) accounts for 1% of all CRC cases and is characterized by the occurrence of hundreds of adenomas that left without intervention progress to cancer in nearly 100% of patients [15]. Although with a lower penetrance the lifetime risk for CRC in hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) is around 80 to 90% [15].

1.1.2. *Natural history*

Colorectal adenocarcinoma arising from the glandular tissues represent over 95% of cancers in the colon and rectum and were the focus of this thesis [16]. Regardless of etiology, CRC are thought to evolve from noncancerous polyps in orderly multistep process slowly over a period of 10-15 years [17]. The so-called adenomatous polyps are the most likely to progress into cancer and commonly found in older groups [18,19]. Approximately one-third to one-half of people will develop colorectal adenomas by the age of 60 years [19,20]. Most adenomas are asymptomatic with fewer than 10% progressing into cancer [18]. The size,

multiplicity, degree of villous component and grade of dysplasia are bevieved to influence the risk of development to CRC [21-24]. Polyps between 1 and 2 cm or greater than 2 cm in diameter have a 10 and 50% probability of malignant transformation, respectively [23].

Furthermore, patients with previous history of colorectal adenomas are at increased risk for the recurrence of adenomas even after removal of those precancerous lesions [25]. Likewise, predictors for colorectal adenoma recurrence include multiple or large adenomas, severe dysplasia, villous componente and adenomas detected in the proximal colon [26]. The rate for the occurence of metachronous adenomas can achieve the 40% to 50% [25]

The adenoma-carcinoma sequence is the phenotypic manifestation of the cumulative effects of acquired molecular events due to loss of genomic stability [27-29]. Briefly, the multiple tumor-associated mutations acquired during colorectal carcinogenesis are driven by at least two major pathways. The chromosomal instability (CIN) is implicated in 60% to 70% of CRCs and includes widespread numeric chromosomal aberrations [30], most frequently observed in chromosome 5q, 18q and 17p and mutation of *KRAS* oncogene [31]. These chromosomes encode the noteworthy *APC*, *DCC* and *TP53* genes, respectively, found to be deregulated in different stages of tumor development [31]. The microsatellite instability (MSI) pathway is characterized by the accumulation of somatic alterations in microsatellite repeat lenght as consequence of aberrant promoter hypermethylation of mismatch repair genes (MMR) [29]. *BRAF* mutations are commonly reported in the context of MSI mainly due to *MLH1* promoter hypermethylation [32]. These entities contribute to the deregulation of important pathways contributing to the hallmarks of cancer [33,34].

1.1.3. *Screening, surveillance and prevention*

Colorectal cancer is particularly suitable of prevention considering its natural history with a long latency period providing an excellent window of opportunity for early detection [17]. Current guidelines endorse several tests and strategies as can be observed in Table 1 [35]. These approaches contrast on their sensitivity to detect different stages of colorectal carcinogenesis with further repercussion on

the interval between examinations, length of observed colon, examination time, prior colon preparation, cost and associated risks [35].

The screening and surveillance of individuals previously diagnosed with colorectal adenomatous polyps is the cornerstone in CRC prevention by effectively reducing CRC incidence and mortality [36,37]. In fact, the United States is the only country presenting a significantly decrease in incidence rates in both genders most attributable to the detection and removal of these colorectal precancerous lesions [38,39]

To date, only faecal occult blood test (FOBT) has been recommended for population-based CRC screening in average-risk males and females aged 50 to 74 years in the European Union [40]. The detection of CRC earlier in more treatable stages has been shown to reduce the risk of death by 15% to 33% [35].

Despite strong evidence supporting the effectiveness of the several tests and strategies, the screening rates for CRC remain low for a meaningful impact in CRC burden. In the United States only half of adult population aged 50 or older reported being screened within recommended intervals [41].

Furthermore, endoscopic surveillance of patients previously diagnosed with colorectal adenomas is recommended considering the high risk of reccurence due to missed or incompletely removed lesions at baseline colonoscopy or to an accelerated tumor growth in apparently normal mucosa that could also account for the development of cancers between colonoscopies [42-46].

The implementation of population-based screening strategies focused primarily on the early detection of CRC and the lower than desirable adherence rate in countries with endoscopic-based CRC screening programmes, emphasize the search for complementary approaches targeting the primary prevention of CRC.

Table 1. Screening tests for colorectal cancer (adapted from [41])

Test	Benefits	Performance & Complexity*	Limitations	Test time interval
Flexible sigmoidoscopy	-Fairly quick -Few complications -Minimal bowel preparation -Minimal discomfort -Does not require sedation or a specialist	Performance: -High for rectum & lower one-third of the colon Complexity: -intermediate	-Views only one-third of colon -Bowel preparation needed -Small risk of infection or bowel tear -Slightly more effective when combined with annual fecal accult blood testing -Colonoscopy necessary if abnormalities are detected	5 years
Colonoscopy	-Examines entire colon -Can biopsy and remove polyps -Can diagnose other diseases -Required for abnormal results from all other tests	Performance: -Highest Complexity: -Highest	-Can miss some polyps and cancers -Full bowel preparation needed -Can be expensive -Sedation of some kind usually needed, necessitating a chaperon -Patient may miss a day work -Highest risl of bowel tears or infections compared to other tests	10 years
Double-contrast barium enema	-Can usually view entire colon -Few complications -No sedation needed	Performance: High Complexity: High	-Can miss some small polyps and cancers -Full bowel preparation needed -Cannot remove polyps -Exposure to low-dose radiation -Colonoscopy necessary if abnormalities are detected	5 years
Computed tomography colonography	-Examines entire colon -Fairly quick -Few complications -No sedation needed -Noninvasive	Performance: High Complexity: Intermediate	-Can miss some polyps and cancer -Full bowel preparation needed -Cannot remove polyps -Exposure to low-dose radiation -Colonoscopy necessary if abnormalities are detected	5 years
Fecal occult blood test	-No bowel preparation -Sampling is done at home -Low cost -Noninvasive	Performance: Intermediate for cancer Complexity: Lowest	-May require multiple stool samples -Will miss most polyps and some cancers -Higher rate of false-positives than other tests -Pre-test dietary limitations -Slightly more effective when combined with flexible sigmoidoscopy every five years -Colonoscopy necessary if abnormalities are detected	Annual
Stool DNA test	-No bowel preparation -Sampling is done at home -Requires only a single stool sample -Noninvasive	Performance: -Intermediate for cancer Complexity: Low	-Will miss most polyps and some cancers -High cost compared with other stool tests -New technology with uncertain nterval between testing -Colonoscopy necessary if abnormalities are detected	Uncertain

* Complexity involves patient preparation, inconvenience, facilities and equipment needed, and patient discomfort.

Chemoprevention is defined as the use of natural, synthetic or biological agents to reverse, suppress or prevent the development or progression tumors in the initial phases of carcinogenesis [47].

The rationale for the development of chemopreventive approaches in CRC prevention arose from observational studies highlighting the long-term use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) in the reduction of CRC incidence and mortality. In 1988, Kune and colleagues [48], in the first population-based case-control study, reported a nearly 50% risk reduction for CRC development in regular aspirin consumers. Thereafter, a large body of epidemiological evidences consistently corroborated the protective role of aspirin in all stages of colorectal carcinogenesis [49,50]. Two trials reported that aspirin also reduces the risk of recurrent adenomas in the context of CRC or previous history of adenomas [51-53]. Baron and colleagues [51], observed a relative risk (RR) of 0.81 (95%CI:0.69-0.96) and 0.59 (95%CI:0.38-0.92) for the recurrence of any adenomas and advanced adenomas, respectively, in patients allocated to low doses of aspirin.

In 2007, Flossmann and Rothwell [49] systematically reviewed relevant observational studies and analyzed the long-term effect of aspirin in two randomized trials with more than 20 years of post-trial follow-up. In the clinical setting, allocation to 300 mg of aspirin daily for a minimum period of 5 years resulted in an effective primary prevention of CRC with a 10 years latency period [49]. Furthermore, the long–term use of aspirin also contributes to a decrease in CRC mortality in five randomized trials [54].

Although effective, the prolonged use of aspirin is associated with serious gastrointestinal side effects that compromised their generalized use in the primary prevention of CRC in populations at average risk [55]. However, and quoting Arder and Levin [56] "…*to ignore the potential benefit of chemoprevention is to continue to accept a higher than necessary death rate from CRC in patient populations that are not fully compliant with screening for colorectal neoplasia*".

A more comprehensive understanding of colorectal carcinogenesis, through the identification of genetic and environmental risk factors, might contribute to CRC prevention by targeting screening and chemoprevention to higher-risk individuals.

1.2. Role of COX-2/PGE_2 pathway in colorectal carcinogenesis: focus on COX-2, 15-PGDH, PGT and MRP4 proteins

Chronic inflammation clearly is an important driver of cancer, by stimulating the proliferation and angiogenesis, as well as, inhibiting apoptosis and immune surveillance, as reviewed by Coussens and Werb [57]. In fact, inflammatory bowel diseases (IBD) such as, ulcerative colitis and Crohn's disease are well-established conditions with increased predisposition for CRC [58]. Furthermore, drugs targeting inflammatory-related molecules were shown to exert anticancer properties, lending further support to the inflammation-cancer cascade [59].

The mechanism underlying the protective actions of NSAIDs is not completely understood but cyclooxygenases (COX) enzymes are their best-known targets [60]. There are at least two COXs isoenzymes identified. COX-1 is a constitutive enzyme ubiquitously expressed and responsible for normal tissue homeostasis, including maintenance of gastric mucosa and regulation of renal blood [61]; whereas COX-2 is an immediate-early response gene normally undetected in most cells but shown to be progressively overexpressed in colorectal adenomas (40 to 50%) and CRC (85%) [62,63] Furthermore, deletion of *COX-2* gene in the $Apc^{Min/+}$ and $APC^{\Delta 716}$ CRC mouse model resulted in a decreased tumor formation [64,65].

The COX enzymes catalyze the rate-limiting step of converting free arachidonic acid (AA) to prostaglandin (PG) G_2, which in turn is reduced to an unstable endoperoxide intermediate, PGH_2 and finally metabolized into five structurally related prostanoids by cell type-specific PG synthases, including PGE_2, PGI_2 and thromboxane A_2 (TxA_2) [66,67].

COX-2-derived prostaglandin E_2 (PGE_2) is the most abundant PG found in colorectal tumors [68]. This bioactive lipid portrays a predominant role during colorectal carcinogenesis by binding to specific transmembrane G-protein-couple cell surface receptors (EP1 to EP4) and activating a plethora of signaling pathways that contribute to most if not all hallmarks of cancer, as reviewed by several authors (see Figure 2) [69,70,71]. In a mouse model for intestinal neoplasia bearing inactivating mutations in the *APC* gene, $Apc^{Min/+}$ stimulation with PGE_2 led to a dramatic increase in the burden of small and large intestinal tumors and significantly enhanced colon tumor incidence and multiplicity in the colon

carcinogen azoxymethane (AOM)-induced mouse cancer model [72,73]. Moreover, in humans the regression of adenomas was particularly noticeable when PGE_2 levels were deeply decreased by NSAIDs [74]. Moreover, increasingly levels of urinary metabolite PGE_2 (PGE-M, 11 alpha-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid) were associated with higher risks for CRC development in a prospective study [75]

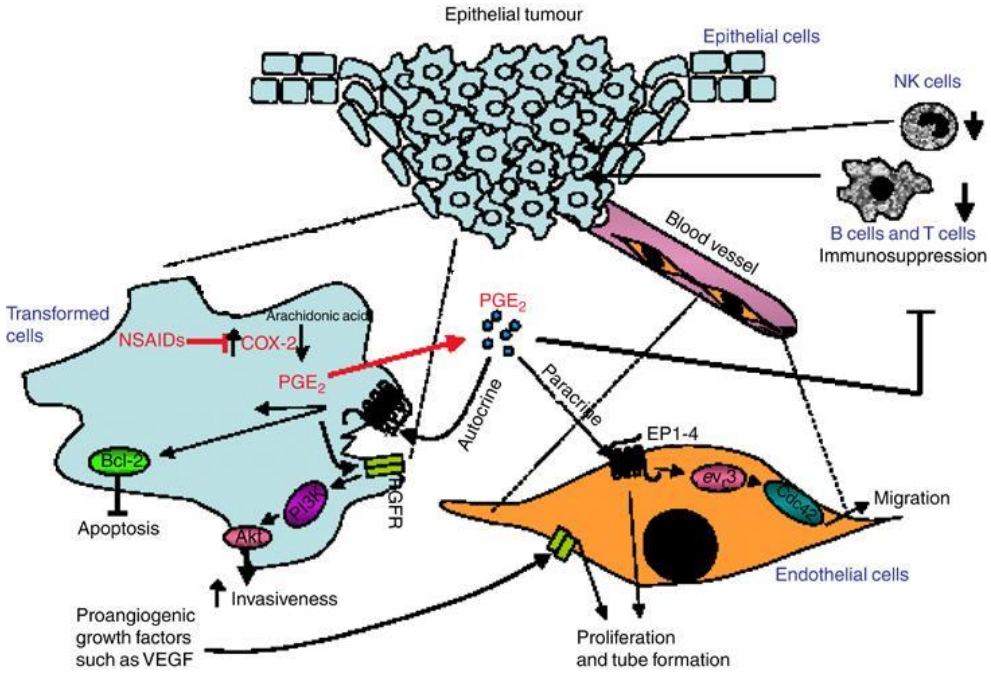


Figure 2**.** Mechanisms of COX-2-derived PGE_2 contribution to tumor development. Adapted from Wang and colleagues [70]. In colorectal tumors, PGE_2, through both autocrine and paracrine regulation can: transactivate EGFR, which results in stimulation of cell migration and invasion through increased PI3K-Akt signaling; induce the production of angiogenic factors, such as, VEGF and bFGF that promote proliferation, migration and vascular tube formation; promote tumor survival by activating PPARδ via PI3K-Akt pathway and inducing antiapoptotic protein expression, like Bcl-2; and modulate immunosurveillance via inhibition of dendritic cell differentiation and T cell proliferation and suppression of antitumor activity of NK cells and macrophages, as reviewed by Wang and colleagues [69,71]. bFGF, basic fibroblast growth factor; COX-2, cyclooxygenase-2; EGFR, epidermal growth factor receptor; NK, natural killer; NSAIDs, nonsteroidal anti-inflammatory drugs; PGE_2, prostaglandin E_2; PI3K, phosphoinositide-3 kinase; PPAR, peroxisome proliferator-activated receptor; VEGF, vascular endothelial growth factor.

A balance between COX-2-dependent biosynthesis and PGE_2 degradation maintains the steady-state cellular levels of PGE_2. It as been suggested that inactivation of PGE_2 in microenvironment is a two-step process [76]. After being synthesized, intracellular PGE_2 is carried through the membrane *via* specific PG efflux transporters, the multidrug resistance-associated protein 4 (MRP4), in an ATP-dependent manner or by simple diffusion and hence their pleiotropic effect on tumor development are triggered [77]. The first step on PGE_2 inactivation requires the carrier-mediated transport by the specific PG transporter (PGT) from the extracellular milieu into the cytoplasm, where the rate-limiting step of PG catabolism is promoted by the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) [76,78]. The 15-PGDH is a natural antagonist of COX-2 pro-carcinogenic effects in colonic mucosa and shown to ubiquitously lost in colorectal tumors [79,80]. Genetic deletion of 15-PGDH was reported to increase tissues levels of PGE_2 promoting colon tumor growth in $Apc^{Min/+}$ [79] and that loss of 15-PGDH expression confers resistance to Celecoxib (COX-2-specific inhibitor) anti-tumor effects [81].

Holla and colleagues [82] reported that PGT expression was decreased in human CRC tissues and in polyps from $Apc^{Min/+}$ mice. Additionally, forced PGT overexpression reduced PGE_2 levels extracellularly and increased its inactive metabolite 15-keto PGE_2 intercellular levels. In turn, MRP4 expression was significantly increased in colorectal tumors compared with normal tissue [82].

The efflux-dominated flow of PG during carcinogenesis as a reflection of an increased expression of COX-2 and MRP4 and down-regulation of 15-PGDH and PGT leads to an accumulation of PGE_2 in the extracellular milieu culminating in the activation of a plethora of pathways that stimulate tumor development [68-71].

1.3. Polymorphisms as risk factors for colorectal cancer

Colorectal cancer has a sizable heritable component. In a large twin study it was estimated that more than one-third of variance in CRC is attributable to heritable factors [83]. Less than 10% of CRC fulfill the criteria for hereditary cancers [14] suggesting that the remaining familiarity might be accounted to a large number of

common, low-penetrance genetic variants each exerting a small influence on risk, mostly represented by single nucleotide polymorphisms (SNP) [84].

Single Nucleotide Polymorphisms (SNPs) are the simplest type of polymorphism and result from a single base mutation, which substitutes one nucleotide for another [85]. Since SNPs are inherited from one generation to the next, less mutable and have high frequency in the genome, they are largely used in the genetic dissection of diseases such as cancer [85].

A decade ago the release of the first human draft allowed a deeper knowledge on the architecture and function of the human genome [86]. Collectively with the HapMap Project, more than 17 million common variants are catalogued in the SNP database (dbSNP; http://www.ncbi.nlm.nih.gov/snp). Due to the high degree of linkage disequilibrium (LD) observed between SNPs within genomic blocks the majority of common genetic variations can be captured by typing a subset of SNPs, mostly referred as tagSNPs [87]. Association studies based on tagSNPs genotyping have, therefore, been proposed as a comprehensive approach to identify causal genetic variation underlying complex diseases and have become an important tool in clinical studies [87].

Not disregarding the importance of underlying cellular phenotypes, the expression levels of many genes naturally differ among individuals. Studies uncovering the genetic basis of variation in gene expression further support the involvement of polymorphisms on the genesis of common diseases as some are expected to influence gene expression [88].

Theodoratou and colleagues [89] recently performed a field synopsis of genetic association studies in CRC. Over 600 studies studies were published reporting 445 polymorphisms in 110 different genes, highlighting the attention this field of research captures.

Our group has previously identified and characterized two genetic variants in inflammatory-related genes as risk biomarkers for CRC occurrence in the Northern Portuguese population [90] (see appendix).

The search for genetic biomarkers in 15-PGDH, MRP4 and PGT coding genes, the *hydroxyprostaglandin dehydrogenase* (*HPGD*), *ATP-binding cassete sub-family c member 4* (*ABCC4*) and *solute carrier organic anion transporter family,*

member 2A1 (*SLCO2A1*) genes, respectively, on colorectal carcinogenesis has been rather neglected [91-95]. Three genetic variants in *HPGD* gene were previously identified as susceptibility biomarkers for CRC development (rs8752, rs2612656 and rs2555639) in the few published studies [91,92].

Most of the efforts have been focused on *COX-2* gene, also known as *prostaglandin-endoperoxide synthase 2* (*PTGS2*). A systematic review on the available data up to this study design is described in the following subchapter [96].

Taken all together, it is reasonable that some genetic polymorphisms in *COX-2/HPGD/ABCC4/SLCO2A1* genes might modulate the susceptibility for the development and recurrence of colorectal tumors, thereby yielding the recognition of individuals at higher risk for this disease.

REFERENCES

[1] Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer 2013. Available from: http://globocan.iarc.fr, accessed on 17/04/2014.

[2] Willett W. The search for causes of breast and colon cancer. Nature 1999;338:389-94.

[3] Reddy B, Engle A, Katsifis S, et al. Biochemical epidemiology of colon cancer: effect of types of dietary fiber on fecal mutagens, acid, and neutral sterols in healthy subjects. Cancer Res 1989;49:4629-35.

[4] Liang PS, Chen TY, Giovannucci E. Cigarette smoking and Colorectal cancer incidence and mortality: systematic review and meta-analysis. Int J Cancer 2009;124(10):2406-15.

[5] Cross AJ, Ferrucci LM, Risch A, et al. A large prospective study of meat consumption and Colorectal cancer risk: an investigation of potential mechanisms underlying this association. Cancer Res 2010;70(6):2406-14.

[6] Centre for Reviews and Dissemination (CRD) UoY. The management of colorectal cancers. Effective Health Care 2004;8:1–12.

[7] Ferrari P, Jenab M, Norat T, et al. Lifetime and baseline alcohol intake and risk of colon and rectal cancers in the European prospective investigation into cancer and nutrition (EPIC). Int J Cancer 2007;121(9):2065-72.

[8] Huxley RR, Ansary-Moghaddam A, Clifton P, et al. The impact of dietary and lifestyle risk factos on risk of colorectal cancer: a quantitative overview of the epidemiological evidence. Int J Cancer 2009;125(1):171-80.

[9] Samad AK, Taylor RS, Marshall T, et al. A meta-analysis of the association of physical activity with reduced risk of colorectal cancer. Colorectal Dis 2005;7(3):204-13.

[10] Kirkegaard H, Johnsen NF, Christensen J, et al. Association of adherence to lifestyle recommendations and risk of colorectal cancer: a prospective Danish cohort study. BMJ. 2010;341:c5504.

[11] Butterworth As, Higgins JP, Pharoah P. Relative and absolute risk of colorectal cancer for individuals with a family history: a meta-analysis. Eur J Cancer 2006;42(2):216-27.

[12] Cottet V, Jooste V, Fournel I, et al. Long-term risk of colorectal cancer after adenoma removal: a population-based cohort study. Gut. 2012;61(8):1180-6.

[13] Bernstein CN, Blanchard JF, Kliewer E, et al. Cancer risk in patients with inflammatory bowel disease: a population-based study. Cancer 2001;92(4):854-62.

[14] Lynch HT and de la Chapelle A. Hereditary Colorectal cancer. N Engl J Med (2003);348810):919-32.

[15] Jasperson KW, Tuohy TM, Neklason DW, et al. Hereditary and familial colon cancer. Gastroenterology (2010);138(6)2044-58.

[16] Steward SL, Wike JM, Kato L, et al. A population-based study of Colorectal cancer histology in the United States, 1998-2001. Cancer 2006;197(5 Suppl):1128-41.

[17] Kelloff GJ, Schilsky RL, Alberts DS, et al. Colorectal adenomas: a prototype for use of surrogate end points in the development of cancer prevention. Clin Cancer Res 2004;10(11):3908:18.

[18] Levine JS and Ahnen DJ. Clinical practice. Adenomatous polyps of the colon. N Engl J Med 2006;355(24):2551-7.

[19] Lieberman DA, Weiss DG, Bond JH, et al. Use of colonoscopy to screen asymptomatic adults for colorectal cancer. N Engl J Med 2000;343(3):162-8.

[20] Schatzkin A, Freedman LS, Dawsey SM, et al. Interpreting precursor studies: what polyp trials tell us about large-bowel cancer. J Natl Cancer inst 1994;86(14):1053-7.

[21] O'Brien MJ, Winawer SJ, Zauber AG, et al: The National Polyp Study: patient and polyp characteristics associated with high-grade dysplasia in colorectal adenomas. Gastroenterology 1990;98:371–9.

[22] Atkin WS, Morson BC, Cuzick J: Long-term risk of colorectal cancer after excision of rectosigmoid adenomas. N Engl J Med 1992;326:58–662.

[23] Muto T, Bussey HJ, Morson BC: The evolution of cancer of the colon and rectum. Cancer 1975;36:2251–2270.

[24] Neugut AI, Jacobson JS, Ahsan H, et al: Incidence and recurrence rates of colorectal adenomas: a prospective study. Gastroenterology 1995;108:402–8.

[25] Yamaji Y, Mitsushima T, Ikuma H, et al. Incidence and recurrence rates of colorectal adenomas estimated by annually repeated colonoscopies on asymptomatic Japanese. Gut 2004;53:568–72.

[26] Ji JS, Choi KY, Lee WC, et al. Endoscopic and histopathological predictors of recurrence of Colorectal adenoma on lowering the miss rate. Korean J Intern med 2009;24(3):196-202.

[27] Fearon Er, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990;61(5):759-67.

[28] Vogelstein B, Kinzler KW: The multistep nature of cancer. Trends Genet 1993;9(4):138-41.

[29] Lengauer C, Kinzler KW, Vogelstein B: Genetic instabilities in human cancers. Nature 1998;396(6712):643-9.

[30 Pritchard CC, Grady WM. Colorectal cancer molecular biology moves into clinical practice. Gut 2011;60:116–29.]

[31] Calvert PM, Frucht H. The genetics of colorectal cancer. Ann Intern Med 2002; 137:603–12.

[32] Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 2006;38:787–93.

[33] Schneikert J and Behrens J. The canonical wnt signaling pathway and its APC partner in colon cancer development. Gut 2007;56:417-25.

[34] Steele RJC, Thompson AM, Hall PA, et al. The p53 tumour suppressor gene.Br J Surg 1998;85:1460–7.

[35] Levin B, Lieberman DA, McFarland B, et al. Screening and Surveillance for the Early Detection of Colorectal Cancer and Adenomatous Polyps, 2008: A Joint Guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. CA Cancer J Clin 2008;58:130–60.

[36] Atkin WS, Edwards R, Kralj-Hans, et al. Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a muiticentre randomized controlled trial. Lancet 2010;375:1624-33.

[37] Zauber AG, Winawer SJ, O'Brien MJ, et al. Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. N Engl J Med 2012;366:687-96.

[38] Edwards BK, Ward E, Kohler BA, et al. Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. Cancer 2010;116:544-73.

[39] Siegel RL, Ward EM, Jemal A. Trends in colorectal cancer incidence rates in the United States by tumor location and stage, 1992-2008. Cancer Epidemiol Biomarkers Prev 2012;21:411-416.

[40]. European Commission. European Guidelines for Quality Assurance in Colorectal Cancer Screening and Diagnosis.-First edition. Segnan N, Patnick J, von Karsa L (eds) 2010.

[41] American Cancer Society. Colorectal Cancer Facts & Figures 2011-2013. Atlanta: American Cancer Society 2011.

[42] Lieberman DA, Rex DK, Winawer SJ, et al. Guidelines for Colonoscopy Surveillance After Screening and Polypectomy: A Consensus Update by the multi-Society Task Force on Colorectal Cancer. Gastroenterology 2012;143:844-57.

[43] Hassan C, Quintero E, Dumonceau JM, et al. Post-polypectomy colonoscopy surveillance: European society of Gastrointestinal Endoscopy (ESGE) Guidelines. Endoscopy 2013;45:842-51.

[44] Rex DK, Cutler CS, Lemmel GT, et al. Colonoscopic miss rates of adenomas determined by back-to-back colonoscopies. Gastroenterology 1997;112:24–8.

[45] 20 Anti M, Armuzzi A, Morini S et al. Severe imbalance of cell proliferation and apoptosis in the left colon and in the rectosigmoid tract in subjects with a history of large adenomas. Gut 2001;48: 238–46.

[46] Robertson DJ, Greenberg ER, Beach M et al. Colorectal cancer in patients under close colonoscopic surveillance. Gastroenterology 2005;129:34–41.

[47] Sporn MB. Approaches to prevention of epithelial cancer during the preneoplastic period. Cancer Res 1976;36:2699–702.

[48] Kune GA, Kune S, Watson LF. Colorectal cancer risk, chronic illnesses, operations, and medications: case control results from the Melbourne Colorectal Cancer Study. Cancer Res 1988;48(15):4399-404.

[49] Flossmann E, Rothwell PM; British Doctors Aspirin Trial and the UK-TIA Aspirin Trial. Effect of aspirin on long-term risk of colorectal cancer: consistence form randomized and observational. Lancet 2007;369(9573):1603-13.

[50] Cole BF, Logan RF, Habali S, et al. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. J Natl Cancer Inst 2009;101(4):256-66.

[51] Baron JA, Cole BF, Sandler RS, et al. A randomized trial of aspirin to prevent colorectal adenomas. N Engl J Med 2003;348:891– 9.

[52] Sandler RS, Halabi S, Baron JA, et al. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. N Engl J Med 2003;348:883– 90.

[53] Gao F, Liao C, Liu L, et al. The effect of aspirin in the recurrence of Colorectal adenomas: a meta-analysis of randomized controlled trials. Colorectal Dis 2009;11(9):893-901.

[54] Rothwell PM, Wilson M, Elwin CE, et al. Lond-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomized trials. Lancet 2010;376(9654):1741-50.

[55] Rodríguez LA and Tolosa LB. Risk of upper gastrointestinal complications among users of traditional NSAIDs and COXIBs in the general population. Gastroenterology 2009;132(2):4498-506

[56] Arber N, Levin B. Chemoprevention of colorectal neoplasia: the potential for personalized medicine. Gastroenterology 2008;134(4):1224-37.

[57] Coussens LM and Werb Z. Inflammation and cancer. Nature 2002;420:860-7.

[58] Krok KL and Lichtenstein GR. Colorectal cancer in inflammatory bowel disease. Curr Opin Gastroenterol 2004;20(1):43-8.

[59] Baumgart DC and Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet 2007;369:1641-57.

[60] Vane JR, Blotting RM. Mechanism of action of nonsteroidal anti-inflammatory drugs. Am J Med 1998;104(3A):2S-8S;

[61] Vane JR, Bakhle YS, Botting RM. Cyclooxygenase 1 and 2. Ann Rev Pharmacol Toxicol 1998;38:97-120.

[62] Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, Dubois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107(4):1183-8.

[63] Gupta RA and Dubois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer. 2001;1(1):11-21.

[64] Chulada PC, Thompson MB, Mahler JF, et al. Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. Cancer Res 2000;4705-8.

[65] Oshima M, Dinchuk JE, Kargman SL, et al. Suppression of intestinal polyposis in APC 716 knockdown mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996;87:803-9.

[66] Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 1971;231(25):232-5.

[67] Funk CD. Prostaglandins and leukotrienes: Advances in eicosanoid biology. Science 2001;294:1871-5.

[68] Rigas B, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. J Lab Clin Med 1993;122(5):518-23.

[69] Wang D, Mann JR, DuBois RN. The role of prostaglandins and other eicosanoids in the gastrointestinal tract. Gastroenterology 2005;128(5):1445-61.

[70] Wang, D. and Raymond, D. Cyclooxygenase-2: a potential target in breast cancer. Seminars in Oncology 2004;31:64-73.

[71] Wang D, Dubois RN. Prostaglandins and cancer. Gut 2006;55(1):115-22.

[72] Wang D, Wang H, Shi Q, et al. Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. Cancer Cell. 2004;6:285–95.

[73] Kawamori T, Uchiya N, Sugimura T, et al. Enhancement of colon carcinogenesis by prostaglandin E2 administration. Carcinogenesis 2003;24:985-90.

[74] Giardiello FM, Casero RA, Jr, Hamilton SR, et al. Prostanoids, ornithine decarboxylase, and polyamines in primary chemoprevention of familial adenomatous polyposis. Gastroenterology. 2004;126:425–31.

[75] Cai Q, Gao YT, Chow WH, et al. Prospective Study of Urinary Prostaglandin E_2 Metabolite and Colorectal Cancer Risk. J Clin Oncol 2006;24:5010-6.

[76] Nomura T, Lu R, Pucci MK, et al. The two-step model of prostaglandin signal termination: *In vitro* reconstitution with the prostaglandin transporter and prostaglandin 15-dehydrogenase. Mol Pharmacol 2004;65:973-8.

[77] Reid G, Wielinga P, Zelcer N, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci USA 2003;100:9244–9.

[78] Tai HH, Ensor CM, Tong M, Zhou H, Yan F. Prostaglandin catabolizing enzymes. Prostaglandins Other Lipid Mediat 2002;68-69:483-93.

[79] Backlund MG, Mann JR, Holla VR, et al. 15-Hydroxyprostaglandin Dehydrogenase is Down-regulated in Colorectal Cancer. J Biol Chem 2005;280(5):3217-23.

[80] Myung SJ, Rerko RM, Yan M, Buchanan FG, Tai HH, Musiek ES, Milne GL, Katkuri S, Dubois RN. 15-Hydroxyprostaglandin dehydrogenase is an in vivo suppressor of colon tumorigenesis. PNAS 2006;103(32):12098-102.

[81] Yan M, Myung SJ, Fink SP, et al. 15-Hydroxyprostaglandin dehydrogenase inactivation as a mechanism of resistance to Celecoxib chemoprevention of cólon tumors. PNAS 2009;106(23):9409-13.

[82] Holla VR, Backlund MG, Yang P, et al. Regulation of Prostaglandin Transporters in Colorectal Neoplasia. Cancer Prev Res 2008;1(2):93-9.

[83] Lichtenstein P, Holm NV, Verkasalo PK, Lliadou A, Kaprio J, Koskenvuo M, Pukala E, Skytthe A, Hemminki K. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med 2000;343(2):78-85;

[84] Balmain A, Gray J, Ponder B. The genetics and genomics of cancer. Nat Genet 2003;33(Suppl): 238–44.

[85] Schork NJ, Fallin D, Lanchbury JS. Single nucleotide polymorphisms and the future of genetic epidemiology. Clin Genet 2000;58(4):250-64.

[86] Naidoo N, Pawitan Y, Soong R, et al. Human genetics and genomics a decade after the release of the draft sequence of the human genoma. Human genomics 2011;5(6):577-622.

[87] Johnson GC, Esposito L, Barratt BJ, et al. Haplotype tagging for the identification of common disease genes. Nat Genet 2001;29:233–7.

[88] Morley M, Molony CM, Weber TM, et al. Genetic analysis of genome-wide variation in human gene expression. Nature 2004;430:743-7

[89] Theodoratou E, Montazeri Z, Hawken S, et al. Systematic Meta-analysis and Field Synopsis of genetic Association Studies in Colorectal Cancer. J Natl cancer Inst 2012;104:1433-57.

[90] Pimentel-Nunes P, Teixeira AL, Pereira C, et al. Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. Dig Liver Dis. 2013 Jan;45(1):63-9.

[91] Thompson CL, Fink SP, Lutterbaught JD, et al. Genetic variation in 15-Hydroxyprostaglandin Dehydrogenase and cólon cancer susceptibility. PLoS one 2013;8(5):e64122.

[92] Hoeft B, Linseisen J, Beckmann L, et al. Polymorphisms in fatty acid metabolismo-related genes are associated with Colorectal cancer risk. Carcinogenesis 2010;31(3):466-72.

[93] Frank B, Hoeft B, Hoffmeister M, et al. Association of hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD) variants and Colorectal cancer risk. Carcinogenesis 2010;32:190-6.

[94] poole E, Hsu L, Xiao L, et al. Genetic variation in prostaglandin E2 synthesis and signaling, prostaglandina dehydrogenase, and the risk of Colorectal adenoma. Cancer Epidemiol Biomarkers Prev 2010;19:547-57.

[95] Edwards TL, Shrubsole MJ, cai Q, et al. A study of prostaglandin pathway genes and interactions with current nonsteroidal anti-inflammatory drug use in Colorectal adenoma. Cancer Prev Res 2012;5:855-63.

[96] Pereira C, Medeiros R, Dinis-Ribeiro M. Cyclooxygenase Polymorphisms in Gastric and Colorectal Carcinogenesis: Are Conclusive Results Available? Eur J Gastroenterol Hepatol 2009;21:76-91.

CHAPTER IA: CYCLOOXYGENASE POLYMORPHISMS IN GASTRIC AND COLORECTAL CARCINOGENESIS: ARE CONCLUSIVE RESULTS AVAILABLE?

Cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis: are conclusive results available?

Carina Pereira[a,b], Rui M. Medeiros[a] and Mário J. Dinis-Ribeiro[b,c,d]

Objective **Cyclooxygenases (COX) are important enzymes not only in the maintenance of mucosal integrity but also in pathological processes, namely in inflammation and tumor development in the gastrointestinal tract. Our goal was to understand whether there is a clear role for *COX* polymorphisms in gastric and colorectal carcinogenesis.**

Methods **A systematic review was conducted on observational studies assessing the involvement of *COX* polymorphisms at the onset of gastric or colorectal lesions, retrieved through a MEDLINE database search by May 2008. The dominant genetic model was assumed for each polymorphism and a random-effect model was used for pooling results.**

Results **Twenty-two studies were retrieved reporting a total of 26 *COX* polymorphisms (nine in *COX1* and 17 in *COX2* genes). Carriers of −1329A, −899C alleles, and *429TT genotype revealed increased risk for gastric cancer [odds ratio (OR)=1.83; 95% confidence interval (CI): 1.07–3.10, OR=2.02; 95% CI: 1.00–4.10 and OR=1.34; 95% CI: 1.06–1.71, respectively). For colorectal lesions, the −899G>C and −1329G>A polymorphisms also showed an increased risk for cancer (OR=1.35; 95% CI: 1.01–1.81 and OR=1.36; 95% CI: 1.11–1.66, respectively). Furthermore, C allele carriers of V102V single nucleotide polymorphisms presented a decreased risk for colorectal adenoma onset (OR=0.77; 95% CI: 0.58–1.03).**

Conclusion **Although further studies, namely cohorts and/or adequately matched case–control studies, are required to unravel the impact of most *COX* polymorphisms, clearly there are evidences that support the involvement of −899G>C and −1329G>A *COX2* polymorphisms in either gastric or colorectal carcinogenesis. These markers could be used to optimize management strategies (follow-up and/or chemoprevention).** *Eur J Gastroenterol Hepatol* **21:76–91 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

European Journal of Gastroenterology & Hepatology 2009, **21**:76–91

Keywords: colon cancer, cyclooxygenases, gastric cancer, meta-analysis, polymorphisms

[a]Molecular Oncology, Portuguese Institute of Oncology, Porto, Portugal, [b]Liga Portuguesa Contra o Cancro - Núcleo Regional do Norte, Porto, Portugal, [c]Gastroenterology Department, Portuguese Institute of Oncology and [d]CINTESIS/Department of biostatistics and Medical Informatics, Porto, Portugal

Correspondence to Mário Dinis-Ribeiro, MD, PhD, Serviço de Gastrenterologia, Instituto Português de Oncologia de Francisco Gentil – EPE, Rua Dr António Bernardino Almeida, Porto 4200-072, Portugal
Tel: +351 22 508 4000 x3348; fax: +351 22 508 4001;
e-mail: mario@med.up.pt

Received 12 February 2008 **Accepted** 13 June 2008

Introduction

Gastrointestinal cancers are amongst the leading causes of cancer mortality. Gastric and colorectal cancers (CRCs), with the second and fourth highest cancer mortalities, respectively, were responsible for 1 229 327 deaths, approximately 18.3% of all cancer-related mortality in 2002 [1].

Several types of studies demonstrated that gastric and colorectal carcinogenesis develops upon a multistep process involving the transformation of normal mucosa to benign precancerous lesions, such as atrophy and intestinal metaplasia (AIM) in gastric adenocarcinoma (GC) (according to Correa's model), [2] and adenomatous polyps in CRC [3,4].

Furthermore, these cancers are complex and multifactorial diseases, emerging from the combined influence of environmental factors such as diet, lifestyle, *Helicobacter pylori* infection, and the individual genetic background [5–8].

Inflammation, nowadays, is a field of highlighted interest in the development and progression of several gastrointestinal cancers [9–11]. Epidemiological and animal data revealed that the use of NSAIDs might reduce the risk of gastric cancer, colorectal polyps, and cancer [12–15]. Although not clearly understood, the ability of NSAIDs to suppress inflammation is strongly credited to the inhibition of cyclooxygenase (COX) enzyme [16,17].

COXs or prostaglandin endoperoxide synthases (PTGS) are rate-limiting enzymes that catalyze the formation of prostaglandins (PG) from arachidonic acid (AA) [18]. COX-1 is constitutively expressed in the majority of tissues and is associated with housekeeping functions like vascular homeostasis and platelet aggregation [19]. The second isoform (COX-2), almost undetectable under normal physiological conditions, is readily induced in response to mitogens, tumor promoters, cytokines, growth factors, stress-inducing agents promoting inflammatory reactions, and tumor development [19–21]. COX-2

0954-691X © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/MEG.0b013e32830ce7ba

overexpression was reported in several common human malignancies, predominantly of the gastrointestinal tract [22,23], including in 85% of CRC and 67% of GC tumors also as in their associated precancerous lesions [24,25]. Therefore, it is not surprising that functional polymorphisms in *COX*s genes have been studied to define their influence in the susceptibility to develop gastrointestinal malignant diseases.

The discovery of single nucleotide polymorphisms (SNPs) as potential biomarkers in early gastrointestinal tumorigenesis [26] has prompted the development of several studies that are often limited by the small statistical power, hindering a clear definition of the impact of those polymorphisms in gastric and CRC development.

Therefore, we conducted a systematic review to unravel the influence of *COX* polymorphisms in gastrointestinal cancers and associated lesions. This could ultimately allow the recognition of individuals at higher risk that may benefit from surveillance programs and/or chemopreventive strategies.

Materials and methods

Type of study

A systematic review was conducted on manuscripts obtained after applying the inclusion and exclusion criteria (see below) to abstracts collected by introducing a specific query in an on line database (PubMed). Selected studies were then characterized in a structured sheet, the quality assessed and the pooled data statistically analyzed.

Search strategy and papers selection

A MEDLINE database (PubMed) search was performed to retrieve papers linking *COX* polymorphisms and risk of gastrointestinal cancers available on line by May 2008, using the following query:

'{[cox OR cox1 OR cox2 OR ptgs1 OR ptgs2 OR 'Cyclooxygenase 2'(MeSH) OR 'Cyclooxygenase 1'(MeSH)] AND [polymorphism OR polymorphisms OR 'Polymorphism, Genetic'(MeSH) OR 'Polymorphism, Single Nucleotide'(MeSH)]} AND {['gastrointestinal cancers'(All Fields) OR 'Gastrointestinal Neoplasms' (MeSH) OR 'Digestive System Neoplasms'(MeSH) OR 'Gastrointestinal Stromal Tumors'(MeSH)] OR [gastric OR 'Stomach' (MeSH)] AND [cancer OR 'Neoplasms'(MeSH) OR 'precancerous lesions' OR 'Precancerous Conditions'(MeSH) OR adenocarcinoma OR atrophy OR dysplasia OR 'Polyps'(MeSH) OR 'Adenomatous Polyps'(MeSH)]} OR 'intestinal metaplasia' OR 'Gastritis, Atrophic'(MeSH) OR {['Esophagus'(MeSH) OR esophageal] AND [cancer OR 'Neoplasms'(MeSH) OR adenocarcinoma OR 'squamous cell carcinoma']} OR 'barrett syndrome'(All Fields) OR 'Barrett Esophagus'(MeSH) OR {['Colon'(MeSH) OR colonic OR colorectal OR intestinal OR bowel OR jejunal OR ileal OR rectal] AND [cancer OR 'Neoplasms'(MeSH) OR 'precancerous lesions' OR 'Precancerous Conditions'(MeSH) OR adenocarcinoma OR 'Polyps'(MeSH) OR 'Adenomatous Polyps'(MeSH)]}'.

One hundred and fifty-one abstracts gathered through this search were then read and the inclusion/exclusion criteria applied independently by two researchers (PC and DRM) (Fig. 1).

Observational studies (case–control and cohort) aimed at assessing the association between *COX* polymorphisms and sporadic gastrointestinal cancers and/or its precancerous lesions were included in this systematic review.

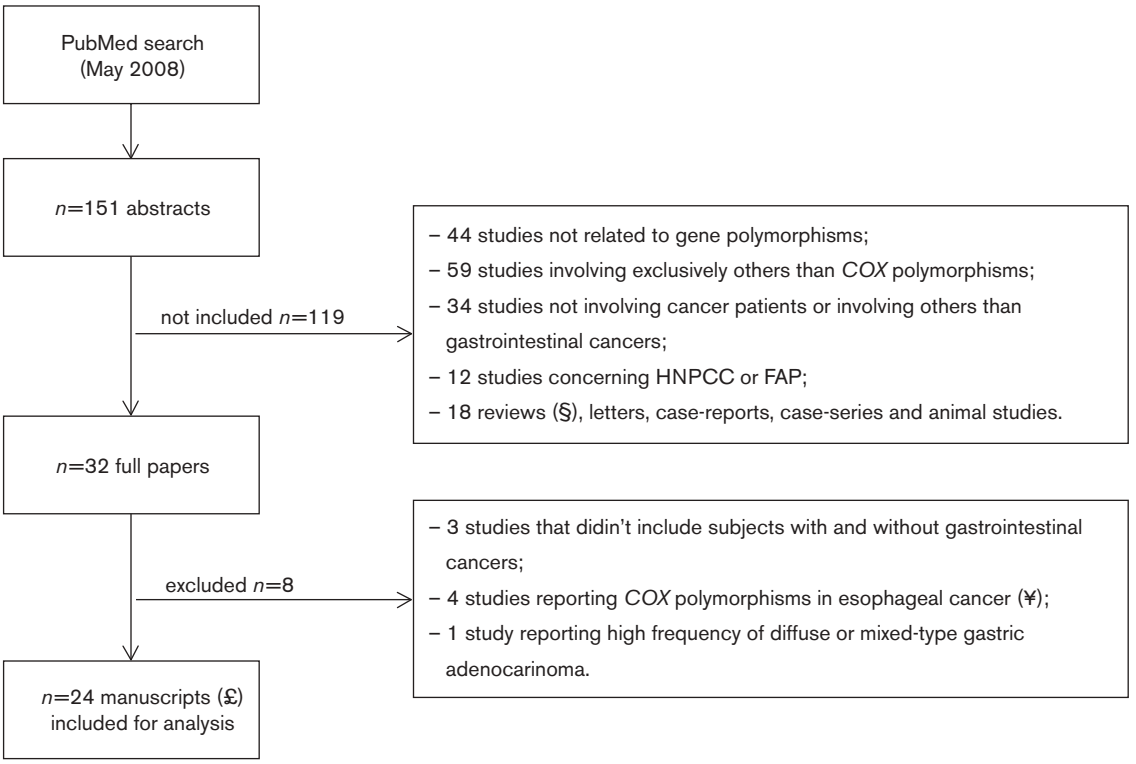
Of the 32 articles meeting the primary criteria [27–58], seven were promptly excluded: four studies investigating esophageal cancer were omitted (two with overlapping data on esophageal squamous cell carcinoma [27,28] and the two studies focusing on esophageal adenocarcinoma examined different *COX2* polymorphims [29,30]); the other three did not include patients with and without gastrointestinal lesions in their studies [i.e. not allowing the odds ratio (OR) estimation] [33,34,58]. We also retrieved one paper published in Chinese without English, French, Spanish, or Portuguese version [31]. To overcome this language barrier, a genotype distribution extraction sheet was sent, by e-mail, to the authors. After exploring full papers another report was excluded. In the study developed by Sitarz *et al.* [57], an extremely high frequency of diffuse and mixed-type GC was noticed (over 60%) and as COX-2 is mainly associated with the intestinal histological-type multistep cascade [59], considering this study in the pooled analysis could disguise the real impact of *COX2* naturally occurring genetic variations in gastric carcinogenesis.

Finally, the reference list of all selected publications and review articles excluded was also checked for additional studies missed on the PubMed search, although no further studies were included.

Data extraction and quality assessment

From each of the included articles the following information was extracted: first author, year of publication, country, ethnicity, study design, histological type, gene and polymorphisms analyzed, number of cases and controls by genotype, representativeness of cases, source of controls, histopathological confirmation of cases and controls, genotyping examination, confounding factors, variables used in statistical adjustments, and evidence of Hardy–Weinberg equilibrium (HWE). If an article reported results from different study populations, those populations were assessed independently [42,47]. From the studies by Koh *et al.* [49] and Tan *et al.* [44], only the pooled CRC's genotype frequencies were extracted, although they had data concerning colon and rectal cancer independently. Furthermore, two overlapping reports were found from Siezen *et al.* [32,40]. Likewise, the paper written by Poole

Fig. 1



Fluxogram for retrieval and papers selection. §, the reference list from reviews was checked for missing papers in the PubMed search. No additional studies were found; ¥, four studies involving esophageal cancer were omitted: two with overlapping data on esophageal squamous cell carcinoma [27,28] and the other two studies focusing on esophageal adenocarcinoma examined different *COX2* polymorphims [29,30]; £, one study was reported in Chinese without Portuguese, English, French, or Spanish version [31]. It was only considered in the statistical analysis; FAP, familial adenomatous polyposis; HNPCC, hereditary nonpolyposis colorectal cancer.

et al. [56] had duplicated data with both studies from Ulrich *et al.* [37,38]. All articles were included for quality assessment, but only the more recent and/or complete article was incorporated in the pooled data [37,38,40]. Articles with stratified results by sex were pooled together [39,50]. Data regarding the use of NSAIDs were assessed either independently or combined [37,38,40,42].

The quality of papers was also independently assessed by two researchers (MR and DRM) based on two published quality score systems, one created by Thakkinstian *et al.* [60] and the STROBE statement [61] (Supplementary data, Table A). Scores ranged from 0 (lowest) to 50 (highest).

Polymorphism characterization

In this systematic review, the different *COX* polymorphisms were designated following the nomenclature proposed by den Dunnen and Antonarakis in 2001 [62]. The different polymorphisms are characterized in Table B of the supplementary data.

Some ambiguity across studies when defining the variant allele for the −1329G>A *COX2* polymorphism was observed. Therefore, considering the functional study developed by Zhang *et al.* [27] we defined the A allele as the variant one since it was shown to increase COX2 transcriptional activity.

Statistical analysis

Pooled variant allele frequencies in control populations

The pooled frequency of each *COX* polymorphism, stratified by ethnicity, was estimated only in the control populations. Heterogeneity across studies was measured through the χ^2 test [degrees of freedom (df) equal to the number of studies minus one].

Hardy–Weinberg equilibrium assessment in control populations

Before the effect estimation of the several *COX* polymorphisms in gastric and colorectal carcinogenesis, the HWE was assessed for all the polymorphisms in each study, whenever unavailable in the original papers, by using the χ^2 test or Fisher's exact test, where appropriate ($n < 5$) (1df). If P value was less than 0.05 then control genotype distributions were assumed to deviate from HWE.

Estimated effect of cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis

For both individual or pooled OR and the corresponding 95% confidence interval (95% CI) estimation, data was inserted on RevMan 4.2.10 statistical program (Copenhage, Denmark) [63]. The dominant model was assumed for every polymorphism and the ORs were estimated under a random-effect model [64]. The heterogeneity statistics was based on Q-value that follows a χ^2 distribution (df equal to the number of studies minus one) [65]. When P value was under 0.05, a statistically significant heterogeneity was assumed among studies. Sources of heterogeneity were appraised by subgroup stratification, based on several study characteristics, like ethnicity and source of control individuals (population or hospital based).

The influence of *COX1* and *COX2* polymorphisms in gastric and colorectal carcinogenesis was assessed independently for each of the following groups: the gastric precancerous lesions group that included patients with AIM; [35,36,53] the gastric cancer patients with lesions as severe as low-grade dysplasia [31,35,36,52,53,55], excluding those with lesions indefinite for dysplasia from the study by Liu *et al.* [36]; the colorectal adenoma group [37–42,54], disregarding patients with hyperplastic polyps reported in both studies by Ulrich *et al.* [37,38] and finally, the CRC group [42–48,51].

Sensitivity analysis and publications bias

By including and excluding studies deviating from HWE sensitivity analysis was performed. Publication bias was assessed through funnel plot asymmetry tests [66].

Results

Studies description

Table 1 summarizes all studies included in this meta-analysis. There were 24 manuscripts [31,32,35–56] reporting 22 studies [31,35–55] included in this review: (i) six studies addressed gastric carcinogenesis [31,35,36,52,53,55], three of which had information not only characterizing gastric cancer patients but also a group with precancerous lesions [35,36,53]. and (ii) 16 studies evaluated the risk of adenoma ($n = 6$) [37–41,54] or CRC ($n = 9$) [43–51] or had data regarding both ($n = 1$) [42].

Nine *COX1* polymorphisms were addressed to uncover their role in the development of gastrointestinal tumors: IVS7 + 14delA (rs3215925), IVS7 – 45T > C (rs3842798), Q41Q (rs3842788), G213G (rs5788), L15_L16del, R8W (rs1236913), P17L (rs3842787), L237M (rs5789) and V481I (rs5794). In *COX2*, 17 polymorphisms were characterized: –1462_1461delTG (rs689464), –1423A > G (rs689465), –1329G > A (rs689466), –899G > C (rs20417), –798A > G, –646C > T (rs20420), –196C > G (rs20424), –125T > G (rs5721), V511A (rs5273), IVS5 – 275T > G (rs20432), IVS7 + 111T > C (rs4648276), V102V (rs5277), G587R (rs3218625), *429T > C (rs5275), *1806A > G (rs4648298), *2291G > A (rs689469), *2430C > T (rs689470).

Studies design

All studies were considered as case–controls, including the four nested case–controls [39,42,48,49]. The median quality was 34.4 points (ranging from 24 [46] to 40 [39]).

Twelve studies used hospital-based cases and controls [35,37,38,40,41,43,45–47,51,52,54] and eight described a population-based design [36,39,44,48–50,53,55]. In the study by Lin *et al.* [42], the risk assessment for colorectal adenoma and cancer pursued a hospital and population-based design, respectively. Only seven studies reported case and control group matching, at least, for age and sex [40–42,45,48,50,55]. All studies adjusted their data for potential confounders [35–42,44–55].

Only 12 studies reported the endoscopic and/or histological confirmation of the cancer-free status for the control groups [36–44,52–54]. Cases and controls were mainly defined through endoscopy procedures, with histological assessment.

Number of participants and addressed populations

A total of 20 576 individuals were studied: 2951 within the precancerous gastric lesions group analysis; 4272 in the gastric carcinoma; 6871 in the colorectal adenoma, and 8172 in the colon cancer groups. Globally, the median number of individuals included was 653, with a minimum of 140 [42] and an upper limit of 2300 participants in the study by Tan *et al.* [44].

Five ethnicities were addressed: eleven studies focused on Caucasian populations [35,37–41,45–48,55], seven on Asiatic [31,36,43,44,49,51,54], three on African–American [42,47,50], one on a Hispanic [53], and one on a Northern Indian population [52]. The African–Americans were only regarded in colon tumors, whereas the Hispanics and an adult population from Northern Indian were exclusively analyzed for onset of gastric lesions.

Allele frequencies in control populations

The genotype distribution of *COXs* polymorphisms following the dominant model and the variant allele frequency is described in Tables 2–5. Briefly, there was homogeneity among study populations for all *COX1* polymorphisms, except for the L237M between Caucasians ($P = 0.012$). The A allele frequency ranged from 2 [40,47,55] to 4% [48]. In *COX2*, heterogeneity was detected across the six studies evaluating the –899G > C SNP in Caucasians ($P = 0.022$) and the pooled C allele frequency ranged from 13 [40] to 22% [35]. Likewise, the six Asiatic studies reporting this polymorphism were also heterogeneous ($P < 0.001$) (2 [31,43,44] to 8% [51]). The *429T > C genetic

Table 1 Study characterization by histological type

Study	Country	Ethnicity	Design	Quality (max 50)	*N*[a]	Representativeness of cases	Source of controls	Histopathological or endoscopic confirmation	Gene (polymorphisms)	Main variables addressed
Gastric precancerous lesions AIM										
Pereira *et al.* [35]	Portugal	Caucasians	Case–control	33	247	Hospital	Blood donors	All cases (EMB)	COX2 (−899G>C)	Adjustment for age and sex
Liu *et al.* [36]	China	Asians	Case–control	33	841	Population	Population (SG/CAG)	All controls and cases (EMB)	COX2 (−899G>C, −1329G>A, G587R)	Adjustment and stratification for age, sex, *H. pylori* infection, smoking, alcohol drinking
Canzian *et al.* [53]	Venezuela	Hispanics	Case–control	39	1863	Population	Population (including CG)	All controls and cases (EMB)	COX1 (Q41Q, G213G, L237M, V481I, IVS7+14delA, IVS7−45T>C); COX2 (IVS5−275T>G, V102V, *429T>C)	Adjustment for age, sex, *H. pylori* infection, smoking, fruit and starchy intake, and other
Gastric adenocarcinoma										
Pereira *et al.* [35]	Portugal	Caucasians	Case–control	33	283	Hospital	Blood donors	All cases (EMB)	COX2 (−899G>C)	Adjustment for age and sex
Liu *et al.* [36]	China	Asians	Case–control	33	816	Population and hospital	Population	All controls and cases (EMB)	COX2 (−899G>C, −1329G>A, G587R)	Adjustment and stratification for age, sex, *H. pylori* infection, smoking, alcohol drinking
Zhang *et al.* [31][b]	China	Asians	Case–control	–	969	–	–	–	COX2 (−1423A>G, −1329G>A, −899G>C)	–
Saxena *et al.* [52]	India	Indians	Case–control	34	303	Hospital	Hospital (NUD)	All controls and cases (E)	COX2 (−899G>C)	Adjustment for age and sex; stratification for *H. pylori* infection
Canzian *et al.* [53]	Venezuela	Hispanics	Case–control	39	1169	Population (Dys)	Population	All controls and cases (EMB)	COX1 (Q41Q, G213G, L237M, V481I, IVS7+14delA, IVS7−45T>C); COX2 (IVS5−275T>G, V102V, *429T>C)	Adjustment for age, sex, *H. pylori* infection, smoking, fruit and starchy intake and other
Hou *et al.* [55]	Poland	Caucasians	Case–control	33	732	Hospital	Population	All cases (nt)	COX1 (L237M, V481I); COX2 (−899G>C, IVS5−275T>G, IVS7+111T>C, V102V, *429T>C, *2430C>T)	Matching for age and sex; adjustment for age, sex, smoking, family history of cancer, diet
Colon adenoma										
Ulrich *et al.* [37][c]	USA	Caucasians	Case–control	36	1142	Hospital	Hospital	All controls and cases (C)	COX1 (R8W, L15–L16del, P17L, L237M)	Adjustment for age, sex, BMI, ethnicity, physical activity, smoking, diet and other; stratification for NSAIDs use
Ulrich *et al.* [38][c]	USA	Caucasians	Case–control	36	1078	Hospital	Hospital	All controls and cases (C)	COX2 (−899G>C)	Adjustment for age, sex, BMI, alcohol drinking, smoking, diet; stratification for NSAIDs use
Ali *et al.* [39]	USA	Caucasians	Nested case–control	40	1505	Population	Population	All controls and cases (S)	COX2 (−798A>G, IVS5−275T>G, *429T>C, −1462_−1461delTG)	Matching for sex; adjustment for age, sex, smoking and NSAIDs use; stratification for sex, smoking, and NSAIDs use
Siezen *et al.* [32][d]	The Netherlands	Caucasians	Case–control	37	787	Hospital	Hospital	All controls and cases (E)	COX1 (R8W, L237M); COX2 (−1329A>G, V102V, *429T>C)	Adjustment for age, sex, alcohol drinking; stratification for diet
Siezen *et al.* [40][d,e]	The Netherlands	Caucasians	Case–control	37	784	Hospital	Hospital	All cases and controls (E)	COX1 (R8W, L237M); COX2 (−1329A>G, -899G>C, V102V, *429T>C)	Matching for age, sex and setting; adjustment for age, gender, duration of smoking; stratification for sex, smoking, family history of cancer, total and fatty fish intake
Gunter *et al.* [41][e]	USA	Caucasians	Case–control	38	475	Hospital	Hospital	All controls (S) and all cases (S or C)	COX2 (−899G>C, V102V, IVS5−275T>G, *429T>C)	Matching for age and sex; adjustment for age, sex, ethnicity
Lin *et al.* [42]	USA	African–Americans	Case–control	35	240[f]/ 140[g]	Hospital	Hospital	All controls and cases [f] (FS) and All controls and cases [g] (C)	COX2 (V511A)	Matching for age, sex, and setting; adjustment for age and sex; stratification by NSAIDs use
Ueda *et al.* [54]	Japan	Asians (men)	Case–control	39	1507	Hospital	Hospital	All controls and cases (C)	COX2 (−1329G>A, −899G>C, *1806A>G)	Adjustment for age, alcohol drinking, smoking BMI, setting
Poole *et al.* [56][c]	USA	Caucasians	Case–control	37	1114	Hospital	Hospital	All controls and cases (C)	COX1 (R8W, P17L); COX2 (−899G>C)	Adjustment for age, sex, BMI, ethnicity, alcohol drinking, smoking, diet, NSAIDs use; stratification for fish intake
Colon adenocarcinoma										
Hamajima *et al.* [43]	Japan	Asians	Case–control		389	Hospital	Hospital	All controls (E) and cases (nt)	COX2 (−899G>C, −125T>C, −163C>G)	
Tan *et al.* [44]	China	Asians	Case–control	30	2300	Hospital	Population	All controls (E) and cases (CP or E)	COX2 (−899G>C, −1329A>G, −1423A>G)	Adjustment for age and sex
Cox *et al.* [45]	Spain	Caucasians	Case–control	35	566	Hospital	Hospital	All cases (nt)	COX2 (−899G>C, V102V, IVS5−275T>G, *429T>C, −1423A>G, *1806A>G, −196C>G, *2291G>A)	Matching for age and sex; adjustment for age and sex

Table 1 ***(continued)***

Study	Country	Ethnicity	Design	Quality (max 50)	N[a]	Representativeness of cases	Source of controls	Histopathological or endoscopic confirmation	Gene (polymorphisms)	Main variables addressed
Landi *et al.* [46]	Spain	Caucasians	Case–control	24	703	Hospital	Hospital	nt	COX1 (V481I)	Adjustment for age and sex
Goodman *et al.* [47]	USA	Caucasians and African–Americans	Case–control	30	511[h]/315[i]	Hospital	Hospital & Population	nt	COX1 (L237M, V481I); COX2 (−646C>T, V511A)	Adjustment for age, sex; stratification for ethnicity
Siezen *et al.* [48]	The Netherlands	Caucasians	Nested case–control	37	603	Population (PPHV)	Population (PPHV)	nt	COX1 (R8W, L237M); COX2 (−1329A>G, V102V, *429T>C, *1806A>G)	Matching for age, sex, and setting; adjustment for age, sex, smoking
Koh *et al.* [49]	China	Asians	Nested case–control	38	1487	Population	Population	All cases, except three (ascertained by death records and clinical evidence)	COX2 (−899G>C)	Adjustment for age, sex, BMI, smoking, alcohol drinking, familial history of colorectal cancer; stratification by diet
Lin *et al.* [42]	USA	African–Americans	Nested case–control	35	396	Population	Population	nt	COX2 (V511A)	Adjustment for age and sex; stratification by NSAIDs use
Sansbury *et al.* [50][e]	USA	African–Americans	Case–control	31	566	Population	Population	nt	COX2 (V511A)	Matching by age and sex; adjustment for age, sex, offset term; stratification for NSAIDs use
Xing *et al.* [51]	China	Asians	Case–control	30	336	Hospital	Hospital	All cases (nt)	COX2 (−899G>C)	Adjustment for age, sex, smoking, alcohol drinking, and BMI; stratification for smoking, alcohol drinking, and BMI

[a]Number of individuals studied.
[b]From this study written in Chinese without English, French, Spanish, or Portuguese version we obtained the genotypes distribution through e-mail contact with authors. The other information was extracted directly from the abstract.
[c]Overlapping data. Only the studies by Ulrich *et al.* [37,38] were comprised in this study (higher number of individuals genotyped).
[d]Duplicated data. Only the more recent and complete study [40] was included in the meta-analysis.
[e]Supplemental information regarding study description was collected from the cited references.
[f]In University of Southern California/Kaiser study.
[g]In University of North Carolina study.
[h]Caucasian population.
[i]African–American population.
AIM, atrophy or intestinal metaplasia; BMI, body mass index; C, colonoscopy; CG, chronic gastritis; CP, coloprotectomy; Dys, dysplasia; E, endoscopy; EMB, endoscopic multiple biopsies; FS, flexible sigmoidoscopy; nt, method not mentioned; NUD, nonulcer dyspepsia; PPHV, project on cardiovascular disease risk factors; S, sigmoidoscopy; SG/CAG, superficial gastritis/chronic atrophic gastritis.

Table 2 Random effects odds ratio (unadjusted) and 95% CI estimated in this analysis following the dominant model of inheritance for gastric precancerous lesions onset

		Cases (%)		Controls (%)			
Polymorphism	First author, year [ref]	Dominant homozygotes	Variant allele carriers	Dominant homozygotes	Variant allele carriers	Variant allele frequency	OR (95% CI)
COX1							
Q41Q (A>G)	Canzian *et al.* [53][a]	752 (93)	53 (7)	957 (92)	84 (8)	0.04	0.80 (0.56–1.15)
G213G (C>A)	Canzian *et al.* [53]	471 (59)	333 (41)	613 (58)	439 (42)	0.24	0.99 (0.82–1.19)
L237M (C>A)	Canzian *et al.* [53][a]	776 (96)	32 (4)	1020 (97)	33 (3)	0.02	1.27 (0.78–2.09)
V481I (G>A)	Canzian *et al.* [53][a]	798 (98)	12 (2)	1038 (99)	12 (1)	0.01	1.30 (0.58–2.91)
IVS7−45T>C	Canzian *et al.* [53]	404 (52)	369 (48)	513 (51)	496 (49)	0.29	0.94 (0.78–1.14)
IVS7+14delA	Canzian *et al.* [53]	472 (61)	307 (39)	618 (60)	406 (40)	0.23	0.99 (0.82–1.20)
COX2							
−1329G>A	Liu *et al.* [36]	106 (26)	308 (74)	105 (25)	322 (75)	0.50	0.95 (0.69–1.29)
−899G>C	Liu *et al.* [36]	373 (90)	41 (10)	384 (90)	43 (10)	0.05	0.98 (0.63–1.54)
	Pereira *et al.* [35]	27 (73)	10 (27)	130 (62)	80 (38)	0.22	0.60 (0.28–1.31)
Pooled OR							0.86 (0.56–1.32)
IVS5−275T>G	Canzian *et al.* [53]	467 (61)	299 (39)	611 (61)	392 (39)	0.22	1.00 (0.82–1.21)
V102V (G>C)	Canzian *et al.* [53][a]	619 (77)	183 (23)	839 (80)	214 (20)	0.11	1.16 (0.93–1.45)
G587R (G>A)	Liu *et al.* [36]	379 (96)	14 (4)	384 (94)	23 (6)	0.03	0.62 (0.31–1.22)
*429T>C	Canzian *et al.* [53]	332 (42)	468 (58)	398 (38)	644 (62)	0.38	0.87 (0.72–1.05)

[a]Contact with authors (through e-mail) was established to recover the discriminated genotypes distribution.
CI, confidence interval; OR, odds ratio.

Table 3 Random effects odds ratio (unadjusted) and 95% CI estimated in this analysis following the dominant model of inheritance for gastric cancer onset

		Cases (%)		Controls (%)			
Polymorphism	First author, year [ref]	Dominant homozygotes	Variant allele carriers	Dominant homozygotes	Variant allele carriers	Variant allele frequency	OR (95% CI)
COX1							
Q41Q (A>G)	Canzian *et al.* [53][a]	107 (95)	6 (5)	957 (92)	84 (8)	0.04	0.64 (0.27–1.50)
G213G (C>A)	Canzian *et al.* [53]	72 (63)	42 (37)	613 (58)	439 (42)	0.24	0.81 (0.55–1.21)
L237M (C>A)	Canzian *et al.* [53][a]	112 (97)	3 (3)	1020 (97)	33 (3)	0.02	0.83 (0.25–2.74)
	Hou *et al.* [55]	292 (96)	13 (4)	402 (97)	14 (3)	0.02	1.28 (0.59–2.76)
Pooled OR							1.13 (0.59–2.15)
V481I (G>A)	Canzian *et al.* [53][a]	113 (97)	3 (3)	1038 (99)	12 (1)	0.01	2.30 (0.64–8.26)
	Hou *et al.* [55]	317 (99)	4 (1)	429 (99)	3 (1)	0.00	1.80 (0.40–8.12)
Pooled OR							2.08 (0.78–5.50)
IVS7−45T>C	Canzian *et al.* [53]	58 (52)	53 (48)	513 (51)	496 (49)	0.29	0.95 (0.64–1.40)
IVS7+14delA	Canzian *et al.* [53]	72 (66)	37 (34)	618 (60)	406 (40)	0.23	0.78 (0.52–1.19)
COX2							
−1423A>G	Zhang *et al.* [31]	283 (88)	40 (12)	592 (92)	54 (8)	0.04	**1.55 (1.01–2.39)[c]**
−1329G>A	Liu *et al.* [36]	73 (19)	316 (81)	105 (25)	322 (75)	0.50	**1.41 (1.01–1.98)[b,c]**
	Zhang *et al.* [31]	32 (10)	291 (90)	136 (21)	510 (79)	0.52	**2.43 (1.61–3.66)[c]**
Pooled OR							**1.83 (1.07–3.10)[c]**
−899G>C	Liu *et al.* [36]	346 (89)	42 (11)	384 (90)	43 (10)	0.05	1.08 (0.69–1.70)[b]
	Zhang *et al.* [31]	288 (89)	35 (11)	620 (96)	26 (4)	0.02	**2.90 (1.71–4.91)[c]**
	Pereira *et al.* [35]	36 (49)	37 (51)	130 (62)	80 (38)	0.22	1.67 (0.98–2.86)
	Saxena *et al.* [52]	14 (23)	48 (77)	171 (71)	70 (29)	0.16	**8.38 (4.34–16.16)[c]**
	Hou *et al.* [55]	210 (72)	80 (28)	288 (70)	121 (30)	0.16	0.91 (0.65–1.27)
Pooled OR							**2.02 (1.00–4.10)[c,d]**
IVS5−275T>G	Canzian *et al.* [53]	72 (67)	36 (33)	611 (61)	392 (39)	0.22	0.78 (0.51–1.19)
	Hou *et al.* [55]	218 (70)	93 (30)	298 (71)	123 (29)	0.16	**1.03 (0.75–1.42)**
Pooled OR							0.93 (0.71–1.21)
V102V (G>C)	Canzian *et al.* [53][a]	83 (72)	32 (28)	839 (80)	214 (20)	0.11	1.51 (0.98–2.3)
	Hou *et al.* [55]	230 (76)	72 (24)	285 (70)	125 (30)	0.16	0.71 (0.51–1.00)
Pooled OR							1.03 (0.49–2.14)[d]
G587R (G>A)	Liu *et al.* [36]	368 (95)	18 (15)	384 (94)	23 (6)	0.03	0.82 (0.43–1.54)[b]
*429T>C	Canzian *et al.* [53]	53 (49)	56 (51)	398 (38)	644 (62)	0.38	**0.65 (0.44–0.97)[c]**
	Hou *et al.* [55]	137 (45)	167 (55)	165 (40)	251 (60)	0.36	0.80 (0.59–1.08)
Pooled OR							**0.74 (0.59–0.94)[c]**
IVS7+111T>C	Hou *et al.* [55]	221 (74)	78 (26)	307 (73)	114 (27)	0.15	0.95 (0.68–1.33)
*2430C>T	Hou *et al.* [55]	289 (94)	19 (6)	399 (98)	10 (2)	0.01	**2.62 (1.20–5.73)[c]**

[a]Contact with authors (through e-mail) was established to recover the discriminated genotypes distribution.
[b]Dysplasia and gastric adenocarcinoma pooled together.
[c]Studies with statistical significant results ($P<0.05$).
[d]Heterogeneity detected across studies ($P<0.05$).
CI, confidence interval; OR, odds ratio.

Table 4 Random effects odds ratio (unadjusted) and 95% CI estimated in this analysis following the dominant model of inheritance for colorectal adenoma onset

		Cases (%)		Controls (%)			
Polymorphism	First author, year [ref]	Dominant homozygotes	Variant allele carriers	Dominant homozygotes	Variant allele carriers	Variant allele frequency	OR (95% CI)
COX1							
L15_L16del	Ulrich *et al.* [37]	510 (98)	11 (2)	616 (99)	5 (1)	0.00	2.66 (0.92–7.70)
R8W (C>T)	Siezen *et al.* [40][a]	334 (90)	39 (10)	339 (87)	53 (14)	0.07	0.75 (0.48–1.16)
	Ulrich *et al.* [37]	445 (85)	76 (15)	539 (87)	82 (13)	0.07	1.12 (0.80–1.57)
Pooled OR							0.94 (0.63–1.40)
L237M (C>A)	Siezen *et al.* [40][a]	300 (96)	14 (4)	332 (97)	12 (3)	0.02	1.29 (0.59–2.84)
	Ulrich *et al.* [37]	493 (95)	28 (5)	585 (94)	36 (6)	0.03	0.92 (0.56–1.53)
Pooled OR							1.02 (0.66–1.56)
P17L	Ulrich *et al.* [37]	451 (87)	70 (13)	527 (85)	94 (15)	0.08	0.87 (0.62–1.22)
COX2							
−1462_−1461delTG	Ali *et al.* [39]	702 (96)	32 (4)	704 (94)	45 (6)	0.03	0.71 (0.45–1.14)
−1329G>A	Siezen *et al.* [40]	22 (6)	349 (94)	16 (4)	377 (96)	0.80	0.67 (0.35–1.30)
	Ueda *et al.* [54]	106 (23)	349 (77)	227 (22)	725 (78)	0.53	0.91 (0.70–1.18)
Pooled OR							0.87 (0.68–1.11)
−899G>C	Siezen *et al.* [40][a,b]	237 (70)	100 (30)	274 (74)	94 (26)	0.13	1.23 (0.88–1.71)
	Gunter *et al.* [41]	151 (72)	59 (28)	141 (72)	55 (28)	0.15	1.00 (0.65–1.54)
	Ulrich *et al.* [38]	344 (70)	150 (30)	405 (69)	179 (31)	0.17	0.99 (0.76–1.28)
	Ueda *et al.* [54]	440 (97)	15 (3)	989 (94)	62 (6)	0.03	**0.54 (0.31–0.97)**[c]
Pooled OR							0.96 (0.74–1.25)
−798A>G	Ali *et al.* [39]	490 (66)	251 (34)	493 (66)	257 (34)	0.19	0.98 (0.79–1.22)
IVS5−275T>G	Ali *et al.* [39]	534 (71)	213 (29)	530 (70)	223 (30)	0.17	0.95 (0.76–1.18)
	Gunter *et al.* [41]	149 (71)	61 (29)	136 (69)	61 (31)	0.16	0.91 (0.60–1.40)
Pooled OR							0.94 (0.77–1.15)
V102V (G>C)	Siezen *et al.* [40][a]	284 (74)	100 (26)	267 (66)	136 (34)	0.19	**0.69 (0.51–0.94)**[c]
	Gunter *et al.* [41]	154 (73)	56 (27)	142 (72)	55 (28)	0.15	0.94 (0.61–1.45)
Pooled OR							0.77 (0.58–1.03)
V511A (T>C)	Lin *et al.* [42][a] USS/K study	113 (95)	6 (5)	109 (90)	12 (10)	0.05	0.48 (0.17–1.33)
	Lin *et al.* [42][a] UNC study	39 (93)	3 (7)	88 (90)	10 (10)	0.05	0.68 (0.18–2.60)
Pooled OR							0.55 (0.24–1.23)
*429A>G	Siezen *et al.* [40]	159 (42)	219 (58)	196 (50)	200 (50)	0.30	**1.35 (1.02–1.79)**[c]
	Gunter *et al.* [40]	30 (14)	180 (86)	18 (9)	179 (91)	0.65	0.60 (0.32–1.12)
	Ali *et al.* [39]	311 (41)	438 (58)	347 (46)	409 (54)	0.33	1.19 (0.97–1.47)
Pooled OR							1.11 (0.82–1.51)
*1806A>G	Ueda *et al.* [54]	451 (99)	4 (1)	1042 (99)	9 (1)	0.00	1.03 (0.31–3.35)

[a]Contact with authors (through e-mail) was established to recover the discriminated genotypes distribution.
[b]Genotype distributions given by the author. These frequencies do not overlap with the ones presented in the original paper.
[c]Studies with statistical significant results ($P<0.05$).
CI, confidence interval; OR, odds ratio; UNC, University of North Carolina study; USC/K, University of Southern California/Kaiser study.

variation also showed heterogeneity among Caucasians ($P < 0.001$) [39–41,45,48,55]. The study developed by Gunter *et al.* [41] revealed a *429C allele distribution outside the ones observed in the other five studies (65% vs. 30–36%, $P < 0.001$). The −1423A>G and −1329G>A were detected at a higher frequency among Caucasians than in Asiatic populations (17 [45] vs. 5% [31,44], for −1423G allele, $P < 0.001$ and 79 [40,48] versus 51% [31,36,44,54] for −1329A allele, $P < 0.001$). With the exception of V511A [47] and −646C>G [47] *COX2* SNPs in Caucasians, and *1806A>G in a Japanese population [54], all the other genetic variations are present, by definition, in at least 1% of the population [67]. Through the different studies, all polymorphisms were in HWE but the IVS5−275T>G *COX2* SNP characterized by Ali *et al.* [39].

Gastric carcinogenesis and *COX* polymorphisms

Fifteen *COX* polymorphisms were assessed for their role in gastric carcinogenesis. In Tables 2 and 3, the random-effect ORs following the dominant genetic model for each polymorphism on the risk of gastric lesions is summarized. No publication bias was evident for *COXs* polymorphisms in gastric lesions.

Risk of gastric precancerous lesions

Estimated risk of *COX1* polymorphisms: only the study by Canzian *et al.* [53] reported the involvement of *COX1* polymorphisms in AIM development. No statistically significant association was observed for any of the six polymorphisms.

Estimated risk of *COX2* polymorphisms: the −899G>C was the only *COX2* polymorphism addressed in more than one study. No heterogeneity between the two studies was observed, although different ethnic populations were studied [35,36]. No modified risk for AIM was noticed for −899C allele carriers. It should be mentioned that three *COX2* SNPs: G587R, V102V, and *429T>C showed association trends for AIM onset.

Table 5 Random effects odds ratio (unadjusted) and 95% CI estimated in this analysis following the dominant model of inheritance for colorectal cancer onset

		Cases (%)		Controls (%)			
Polymorphism	First author, year [ref]	Dominant homozygotes	Variant allele carriers	Dominant homozygotes	Variant allele carriers	Variant allele frequency	OR (95% CI)
COX1							
R8W (C>T)	Siezen *et al.* [48][a]	168 (84)	33 (16)	335 (86)	56 (14)	0.08	1.18 (0.74–1.88)
L237M (C>A)	Siezen *et al.* [48][a]	182 (96)	8 (4)	348 (92)	31 (8)	0.04	0.49 (0.22–1.10)
	Goodman *et al.* [47] (Caucasians)	161 (90)	17 (10)	309 (96)	14 (4)	0.02	**2.33 (1.12–4.85)[b]**
	Goodman *et al.* [47] (African–Americans)	113 (99)	1 (1)	184 (96)	7 (4)	0.02	0.23 (0.03–1.92)
Pooled OR (Caucasians)							1.08 (0.23–4.98)
V481I (G>A)	Landi *et al.* [46]	280 (99)	3 (1)	265 (98)	5 (2)	0.01	0.57 (0.13–2.40)
	Goodman *et al.* [47] (Caucasians)	173 (98)	3 (2)	324 (98)	6 (2)	0.01	0.94 (0.23–3.79)
	Goodman *et al.* [47] (African–Americans)	115 (100)	0 (0)	196 (99)	1 (1)	0.00	–
Pooled OR (Caucasians)							0.73 (0.27–2.00)
COX2							
−1423A>G	Cox *et al.* [45]	201 (73)	76 (27)	181 (70)	78 (30)	0.17	0.88 (0.60–1.28)
	Tan *et al.* [44]	914 (91)	86 (9)	1180 (91)	120 (9)	0.05	0.93 (0.69–1.24)
Pooled OR							0.91 (0.72–1.14)
−1329G>A	Siezen *et al.* [48]	10 (5)	186 (95)	20 (5)	371 (95)	0.79	1.00 (0.46–2.19)
	Tan *et al.* [44]	178 (18)	822 (82)	300 (23)	1000 (77)	0.50	**1.39 (1.13–1.70)[b]**
Pooled OR							**1.36 (1.11–1.66)[b]**
−899G>C	Cox *et al.* [45]	150 (68)	70 (32)	170 (66)	87 (34)	0.19	0.91 (0.62–1.34)
	Koh *et al.* [49][a]	273 (88)	37 (12)	1067 (91)	110 (9)	–	1.31 (0.89–1.95)
	Hamajima *et al.* [43]	140 (95)	8 (5)	230 (95)	11 (5)	0.02	1.19 (0.47–3.04)
	Tan *et al.* [44]	919 (92)	81 (8)	1237 (95)	63 (5)	0.02	**1.73 (1.23–2.43)[b]**
	Xing *et al.* [51]	119 (87)	18 (13)	169 (85)	30 (15)	0.08	0.85 (0.45–1.60)
Pooled OR							1.21 (0.90–1.61)
Pooled OR (Asians)							**1.35 (1.01–1.81)[b]**
−646C>T	Goodman *et al.* [47][a] (Caucasians)	175 (99)	1 (1)	331 (100)	1 (0)	0.00	1.89 (0.12–30.42)
	Goodman *et al.* [47][a] (African–Americans)	104 (91)	10 (9)	181 (91)	17 (9)	0.04	1.02 (0.45–2.32)
Pooled OR							1.08 (0.49–2.36)
−196C>G	Cox *et al.* [45][a]	280 (97)	10 (3)	263 (98)	6 (2)	–	1.57 (0.56–4.37)
−125T>G	Hamajima *et al.* [43]	141 (97)	5 (3)	230 (97)	7 (3)	0.01	1.17 (0.36–3.74)
IVS5−275T>G	Cox *et al.* [45]	187 (64)	103 (36)	174 (64)	97 (36)	0.20	0.99 (0.70–1.40)
V102V (G>C)	Cox *et al.* [45]	180 (62)	110 (38)	183 (67)	89 (33)	0.18	1.26 (0.89–1.78)
	Siezen *et al.* [48]	142 (70)	61 (30)	287 (72)	111 (28)	0.15	1.11 (0.77–1.61)
Pooled OR							1.19 (0.92–1.53)
V511A (T>C)	Goodman *et al.* [47][a] (Caucasians)	177 (100)	0 (0)	329 (100)	0 (0)	0.00	–
	Goodman *et al.* [47][a] (African–Americans)	109 (95)	6 (5)	186 (93)	14 (7)	0.04	0.73 (0.27–1.96)
	Lin *et al.* [42][a]	129 (93)	9 (7)	237 (92)	21 (8)	0.04	0.79 (0.35–1.77)
	Sansbury *et al.* [50]	223 (93)	17 (7)	292 (90)	34 (10)	0.05	0.65 (0.36–1.20)
Pooled OR							0.71 (0.46–1.09)
*429A>G	Cox *et al.* [45][a]	140 (48)	150 (52)	126 (47)	145 (53)	0.31	0.93 (0.67–1.30)
	Siezen *et al.* [48]	97 (48)	103 (52)	190 (49)	198 (51)	0.30	1.02 (0.72–1.43)
Pooled OR							0.97 (0.77–1.23)
*1806A>G	Cox *et al.* [45][a]	257 (91)	24 (9)	258 (96)	10 (4)	–	**2.41 (1.13–5.14)[b]**
	Siezen *et al.* [48][a]	194 (97)	5 (3)	368 (95)	21 (5)	0.03	0.45 (0.17–1.22)
Pooled OR							1.08 (0.21–5.56)[c]
*2291G>A	Cox *et al.* [45][a]	245 (92)	20 (8)	241 (96)	10 (4)	–	1.97 (0.90–4.29)

[a]Contact with authors (through e-mail) was established to recover the discriminated genotypes distribution.
[b]Studies with statistical significant results ($P<0.05$).
[c]Heterogeneity detected across studies ($P<0.05$).
CI, confidence interval; OR, odds ratio.

Risk of gastric adenocarcinoma

Estimated risk of *COX1* polymorphisms: the two studies evaluating the L237M and V481I *COX1* polymorphisms were homogeneous, although reporting two different ethnical populations [53,55]. No statistically significant results were observed.

Estimated risk of *COX2* polymorphisms: considering only functionally expected polymorphisms (see table B of the Supplementary data), the analysis of −899G>C revealed heterogeneity across studies that could not be explained even after data stratification by ethnicity. A 2.02-fold impact was observed for −899C allele carriers (95% CI: 1.00–4.10, $P_{\text{heterogeneity}} < 0.001$). Likewise, the −1329G>A COX2 polymorphism conferred an increased susceptibility of 1.83 (95% CI: 1.07–3.10) in A allele carriers and of 2.18 among AA genotype carriers (95% CI: 1.36–3.49) for GC development. Interestingly, not only

were these two polymorphisms associated with GC in normal individuals, but also, when pooled together, seemed to potentiate the progression into malignant lesions in patients with AIM (OR = 1.47; 95% CI: 1.00–2.16) (Fig. 2). The *429T > C analysis revealed that C allele carriers were more protected for GC onset (OR = 0.74; 95% CI: 0.59–0.94). Furthermore, when addressing the risk-associated genotypes of the three polymorphims mentioned above, (−1329A allele, −899C allele, and *429TT genotype), the pooled analysis revealed an increased susceptibility for GC (OR = 1.77; 95% CI: 1.25–2.50, $P_{\text{heterogeneity}} < 0.001$) (Fig. 2). The lack of homogeneity, not explained after an ethnicity-based subanalysis, was dissolved within population-based studies (OR = 1.21; 95% CI: 1.01–1.46) [36,53,55]. For the nonfunctional expected V102V polymorphism the two reported studies seemed to be heterogeneous ($P_{\text{heterogeneity}} = 0.007$), showing strong opposing trends that might be explained as both studies were developed in different ethnical populations. The −1423A > G and *2430C > T noncoding region polymorphisms, addressed only once by Zhang *et al.* [31] and by Hou *et al.* [55], respectively, revealed an increased risk of GC.

Colorectal carcinogenesis and *COX* polymorphisms

A total of 19 *COX* polymorphisms were considered throughout 16 studies [37–51,54] assessing their impact on colorectal carcinogenesis. Five were identified within the coding region of *COX1* and 14 throughout *COX2* gene. The random-effect ORs, under the dominant genetic model, for the onset of colorectal lesions are characterized in Tables 4 and 5. We did not observe any obvious publication bias for either *COX1* or *COX2* polymorphisms in colorectal tumors.

Risk of colorectal adenoma

Estimated risk of *COX1* polymorphisms: the studies by Ulrich *et al.* [37] and Siezen *et al.* [40] addressed, altogether, four *COX1* polymorphisms (L15_L16del, R8W, P17L and L237M) in colorectal adenoma. For the functional expected polymorphisms, R8W, P17L, and L237M polymorphisms [68–71], no genetic effect was observed. The L15_L16del *COX1* polymorphism, although characterized only once in the study by Ulrich *et al.* [37], it seemed to increase the risk of colorectal adenoma, although not reaching the significance level.

Estimated risk of *COX2* polymorphisms: nine *COX2* polymorphisms (−1462_−1461delTG, −1329G > A, −899G > C, −798A > G, IVS5−275T > G, V102V, V511A, *429T > C, and *1806A > G) were appraised for colorectal adenoma in a total of six studies [38–42,54]. Only the pooled data for the V102V and V511A suggested a protective trend for colorectal adenoma. Excluding the study by Gunter *et al.* [41] from the pooled analysis of *429T > C *COX2* polymorphism, (C allele frequency very different from all the others in the control populations) we detected an OR of 1.25 (95% CI: 1.06–1.47) in C allele carriers. No influence on adenoma onset susceptibility was noticed for the −899G > C and −1329G > A *COX2* polymorphisms. The sensibility was tested by omitting the study by Ali *et al.* [39] from the IVS5−275G > T analysis. No change in the SNP behavior was observed.

Risk of colorectal cancer

Estimated risk of *COX1* polymorphisms: three studies characterized *COX1* polymorphisms [46–48]. No gene–disease association was noticed for both R8W and V481I *COX1* coding region SNPs. Contradictory data were presented in the individual studies assessing L237M SNP in Caucasians [47,48]. Owing to the limited number of studies we were not able to identify the source of heterogeneity among studies ($P_{\text{heterogeneity}} = 0.006$).

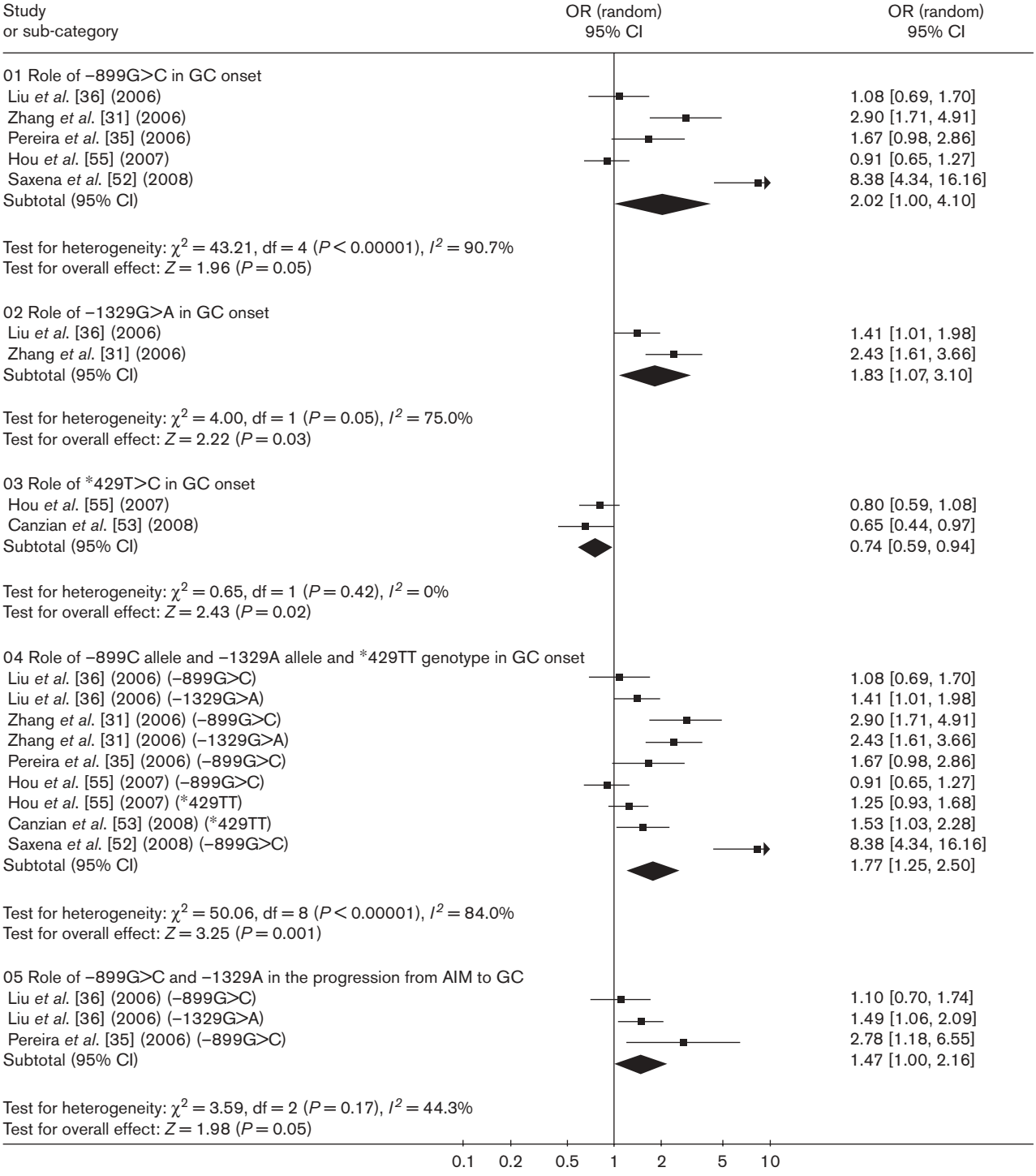
Estimated risk of *COX2* polymorphisms: nine studies were conducted in Caucasian, African–American, or Asiatic populations, gathering a total of 12 *COX2* polymorphisms examined [42–45,47–51]. The overall random-effect OR for −899G > C polymorphism was 1.21 (95% CI: 0.90–1.6). This value achieved statistical significance in Asiatic populations (OR = 1.35; 95% CI: 1.01–1.81) [43,44,49,51]. A 1.36-fold increased risk of CRC development was also observed for the −1329G > A polymorphism (95% CI: 1.11–1.66). The *429T > C polymorphism which was associated with increased risk of colorectal adenoma, did not seem to play any role in the development of CRC. Strong heterogeneity was detected across the two studies [45,48] enrolled involving *1806A > G polymorphism ($P_{\text{heterogeneity}} = 0.004$). Several study characteristics could be used to explain this lack of homogeneity, but the limited number of studies restricted the interpretation of this heterogeneity. The V511A *COX2* polymorphism, only identified in African–Americans, showed a protective tendency for CRC. All the other genetic variations did not alter the susceptibility to developed CRC, although the *2291G > A SNP, assessed once by Cox *et al.* [45], showed a strong increased risk trend for cancer.

No single study addressed the CRC risk onset in colorectal adenoma patients.

Discussion

In November 2002, Lin *et al.* [42] reported the first study addressing *COX* polymorphisms in the susceptibility for CRC onset. Nearly 6 years later, more than 20 studies were developed gathering a total of 26 *COX* polymorphisms appraised in gastrointestinal tumors: nine in *COX1* and 17 in *COX2* genes [31,35–55]. Now, do we have conclusive results that could lead to clinical reasoning or research?

Fig. 2



Forest plot describing the random effect ORs and 95% CI from studies assessing the association between *COX2* functional expected polymorphisms in gastric carcinogenesis: (01) role of −899G>C polymorphism in gastric cancer (GC) onset; (02) role of −1329G>A polymorphism in GC onset; (03) role of *429T>C polymorphism at GC onset; (04) role of −899C, −1329A alleles and *429TT genotype pooled together in GC development and (05) role of −899G>C and −1329G>A polymorphisms in GC onset in patients with atrophy and/or intestinal metaplasia (AIM). All analysis followed the dominant genetic model. I^2 and P value for χ^2 of heterogeneity is reported for each group analysis. CI, confidence interval; OR, odds ratio.

Role of *COX* polymorphisms in gastric carcinogenesis

The different roles that COX-1 and COX-2 enzymes portray, COX-1 associated with housekeeping functions and COX-2 with inflammation and tumor development [19], may explain the effort disparity to assess *COX1* or *COX2* polymorphisms in gastric and colorectal tumors. COX-1 enzyme in stomach is involved in the protection and maintenance of gastric mucosa [19]; therefore it would be interesting to appraise the involvement of *COX1* polymorphisms in the development of gastric lesions, since it was only assessed in two studies [53,55].

In gastric carcinogenesis, polymorphisms in *COX2* gene seemed to differently influence the genetic susceptibility according to the type of gastric lesions assessed (AIM or GC). Despite the publishing of four papers since last year [52,53,55,57], the restricted number of studies included in this analysis ($n = 6$) [31,35,36,52,53,55] only allowed us to draw some remarks and not strong conclusions. Two of the most studied polymorphisms in the promoter region of *COX2* gene ($-1329G > A$ and $-899G > C$) seemed to be associated with susceptibility for gastric cancer onset.

The $-1329A$ allele was associated with a 1.83-fold increased risk of gastric cancer in approximately 1800 Asiatic individuals. In the study carried out by Liu *et al.* [36], the increased susceptibility was even higher in individual carriers of $-1329AA$ genotype positives for *H. pylori* infection (OR = 3.88; 95% CI: 1.46–10.34) or smokers (OR = 7.02; 95% CI: 2.19–22.48). Furthermore, although not addressed in the original study, this polymorphism also seemed to be involved in the development of gastric cancer in patients with intestinal metaplasia (OR = 1.49; 95% CI: 1.06–2.09). All these associations can be biologically supported, because the $-1329A$ allele creates a core recognition sequence for the c-MYB nuclear transcription factor resulting in higher transcription activity of *COX2* as it was proved in the study by Zhang *et al.* [27]. Further studies focusing different ethnical population are important to understand whether this polymorphism behavior is ethnic-specific, as the only study addressed in adult Caucasians [48] did not show any impact on gastrointestinal cancer onset. These should also address several confounding factors like age, sex, *H. pylori* infection, and smoking status.

The most studied *COX2* genetic variation, $-899G > C$, revealed an increased risk behavior associated with the development of GC in the normal population (OR=2.02; 95% CI: 1.00–4.10), although strong heterogeneity was reported that could not be interpreted even after ethnicity and type of controls stratification. From the study by Liu *et al.* [36], we defined individuals with superficial gastritis and chronic atrophic gastritis as belonging to the nonlesions control group. This may not be the best approach since these lesions might already have some resemblances with malignant tumors, thus explaining the nonassociation result observed in this individual study. Another study worth mentioning is the one by Saxena *et al.* [52] where we observed a nine-fold increased risk of GC in a Northern Indian population. This value was significantly higher than all other ethnical populations addressed, suggesting that the contribution of genetic polymorphims may be dependent on the population being studied, as well as on several environmental and dietary factors that influence that population [72]. The molecular mechanism and the biological impact of this polymorphism in cancer development are surrounded by controversy. First characterized by Papafili *et al.* [73] is recognized as functional polymorphism because the transversion from a guanine (G) to a cytosine (C) in the promoter region of *COX2* gene might inhibit the binding of the Sp1 positive transcription factor resulting in a reduction of the promoter activity. In contrast, Szczeklik *et al.* [74] reported that monocytes from $-899CC$ genotype carriers had a 10-fold increase in the production of PG. In addition, Zhang *et al.* [27] also observed that heterozygous individuals seemed to have a higher *COX2* mRNA expression although not being statistically significant. The latter results could be explained as the $-899C$ allele, besides eliminating a Sp1 recognition binding site, also creates and E2F homology binding region, based on bioinformatic programmes, that could lead to a higher transcription activity [27,74]. All of these findings suggest that different cell types under different physiological conditions could determine the $-899G > C$ behavior by the binding of specific nuclear proteins to the promoter region [31]. Therefore, further functional studies in gastric tumors are required to elucidate the molecular mechanism involving the $-899C$ allele in gastric carcinogenesis. Unlike most of the SNPs addressed, the $*429T > C$ *COX2* SNP seemed to play a protective role in both AIM and GC development. When pooling all these three polymorphisms with functional impact on the genetic susceptibility, according to the risk-associated genotypes, increasing the number of studies involved, we observed a nearly two-fold increased risk of gastric cancer onset. Although this warrants further studies, future investigations should focus on the combined influence, haplotype analysis of the $-1329G > A$, $-899G > C$, and $*429T > C$ *COX2* polymorphisms. Besides playing a role in gastric cancer onset in normal adults without expected gastric lesions, the $-899G > C$ and $-1329G > A$ SNPs (pooled together) also showed a gene–disease interaction in patients with AIM. If confirmed by further researches, these polymorphims could allow the identification of higher-risk individuals for gastric cancer development that may beneficiate from chemopreventive and/or follow-up strategies.

Role of *COX* polymorphisms in colorectal carcinogenesis

We can safely say that CRC is one of the most studied models for unraveling the role of COX enzymes in several key steps of carcinogenesis [75]. Nowadays, it is well established that COX-2 plays a pivotal role in early

colorectal carcinogenesis and that the use of NSAIDs, like aspirin, is associated with decreased risk for adenoma and CRC onset and recurrence [76–79].

All the polymorphisms in *COX1* gene were investigated in over 1000 individuals. Only the L15_L16del had any impact on the genetic susceptibility presenting a strong increased risk trend for colorectal adenoma onset in approximately 1200 Caucasians in a hospital-based study carried out by Ulrich *et al.* [37]. Curiously, this increased susceptibility is even more evident in nonregular users (OR = 8.58; 95% CI: 1.07–68.94) and annulled in regular users of NSAIDs (OR = 1.08; 95% CI: 0.24–4.87), confirming that at least part of the anti-inflammatory tumor protective effects of NSAIDs are because of COX inhibition [16]. The remaining polymorphisms expected to have an impact on COX-1 function, following the sequence homology-based software programmes predictions [68,69], did not show any association. An interesting heterogeneity was detected among the two Caucasian studies addressing the L237M polymorphism [47,48] revealing opposing estimates. Owing to the restricted number of studies we did not have the capacity to scrutinize the characteristic that could explain this heterogeneity, as they diverge in several features (demographic origin, study design, number of participants). We will have to wait for further studies to unravel the true meaning of this SNP in CRC development.

COX-2 is the inducible isoform of COX enzymes [19]. Unlike COX-1 that is constitutively expressed in most tissues, COX-2 expression is mainly regulated at transcription level, although posttranscriptional level regulation (*COX2* mRNA stability) also seems to influence COX-2 expression [80,81]. *COX2* promoter region has several recognition sites for nuclear proteins, including Sp1, c-MYB, NF-κB, AP1, TATA [80]. Therefore, it is not surprising that eight of the fourteen *COX2* polymorphisms analyzed for colorectal lesions onset belong to the promoter region (−1462_−1461delTG, −1423A > G, −1329G > A, −899G > C, −798A > G, −646C > T, −196C > G, −125T > G), one to the intronic region (IVS5−275T > G), two to the coding regions (V102V and V511A) and three to the 3′ untranslated region (*429T > C, *1806A > G, *2291G > A). Seven *COX2* polymorphisms, −1329G > A, −899G > C, IVS5−275T > G, V102V, V511A, *429T > C and *1806A > G, were investigated in both adenoma and CRC susceptibility, and all of them, except for the IVS5−275T > G and V511A, seemed to have a histological type-dependent behavior.

The V511A polymorphism, identified only in African–Americans, revealed a nonsignificant histological-type independent protection for colorectal tumors. This lack of association was possibly due to the low frequency of 511A variant (5%). The *in vitro* functional characterization of this polymorphism, that leads to an amino acid change close to COX-2 active site, did not revealed any allele differences in the enzyme kinetic parameters ($V_{máx}$ and K_m) or stability for arachidonic acid utilization [42] Nevertheless, this absence of functional differences between alleles could be limited by conditions *in vitro*, as mentioned by Lin *et al.* [42].

The pooled analysis for the −899G>C *COX2* polymorphism revealed an increased risk association for CRC development in Asiatic adults, although this was not noticed in all individual studies [43,51]. Nevertheless, these studies had a low number of participants (∼350) and as the frequency of this polymorphism is very low in Asiatic populations (2 to 8%), the lack of association may represent low statistical power and confidence level. Likewise, the −1329G > A genetic variation also revealed an increased risk of CRC, although it was considered only in the study by Tan *et al.* [44] which gathered a total of 2300 Asiatic individuals following a population-based design ensuring a statistically significant result. In Caucasians, we did not have enough statistical power to detect any association for either polymorphism. Both polymorphims seemed to have the same increased risk behavior, independent of the tumor locations, but only associated with the more severe forms of gastrointestinal lesions (GC and CRC).

In contrast, the *429T > C and V102V *COX2* polymorphisms seemed to influence the development of colorectal lesions in early stages of carcinogenesis. The *429T > C SNP, associated with protection for GC development, in colorectal lesions exposed a 1.25-fold increased risk of adenoma onset in Caucasians, suggesting an organ-specific involvement. The thymine (T) to cytosine (C) exchange in an AU-rich elements region, known to influence mRNA degradation [81], could enhance the stability of mRNA transcripts that could ultimately lead to an increased PG production [39,82]. This biologic assumption seems to support our outcome in colon, but as, so far, no study has functionally characterized this SNP we can only wait for future evidences. Such a clear association for adenoma development was not detected but instead a protective trend was noticed with the synonymous V102V polymorphism. Further and larger studies are necessary to elucidate this relationship.

The low-frequency *1806G allele identified in the 3′ untranslated region of exon 10 is believed to have an impact on mRNA *COX2* stability through the addition of some poly-A tail to the mRNA, generating a longer and more stable mRNA [45]. Conflicting results were observed between the two individual studies [45,48] carried out with this polymorphism. Owing to the limited number of studies we were not able to identify the source of heterogeneity, although they only seemed to diverge in one feature. The study by Cox *et al.* [45] had a hospital-

based design and in contrast the study by Siezen *et al.* [48] followed a population-based one. Before any remarks could be drawn, further larger epidemiological studies, also as functional tests, are recommended to elucidate the nature of *1806G allele in CRC.

A systematic review can be a resourceful tool in detecting an association that could otherwise remain masked in the studies of a small number participants [83], especially in those evaluating rare allele frequency polymorphisms. Nevertheless, these results should be interpreted bearing in mind the limitations encountered in this analysis. Firstly, the elevated number of *COX* polymorphisms addressed and the lack of genotype frequency information for each one in each of the studies did not allow the estimation of the best genetic model of inheritance to follow. Therefore, we assumed the dominant model for every SNP. For some polymorphisms this model might not be the most suitable to allow a clear assessment of the gene-disease interaction [83]. Secondly, for most polymorphisms we were not able to address the sources of heterogeneity when detected among studies, and to perform subgroup stratifications analysis, because of the limited number of published studies. Thirdly, in at least one study there was a clear age gap between cases and controls. The control group was younger than the patients, meaning that possibly there are participants that could develop cancer before reaching the median age of cases in the control group. Fourthly, several studies did not confirm the absence of lesions in the control group by any technique and several others did not mention the method of choice. The last two drawbacks could ultimately lead to an underestimation of the overall polymorphism effect. Finally, this systematic review was based on unadjusted data, as the genotype information stratified for the main confounding variables was not available in the original papers and also the confounding factors addressed across the different studies were variable. The adjusted estimates could give more precise and strong associations, as they reduce the impact of possible confounding factors.

In future studies several requirements should be fulfilled, concerning not only the study design but also the reporting of data: a sample size estimation based on the genotype distribution should be carried out, especially for low-frequency alleles; the inclusion and exclusion criteria ought to be stated clearly in the reporting manuscript; the control group should represent the same source populations as cases and the main characteristics, age, and sex, should be matched between the two groups; to ensure the correct classification, all participants should be screened for possible gastric or colorectal lesions; this information alongside the validated technique applied should be facilitated when reporting the study; the selection of participants as well as the genotyping examination should be performed by blind personnel; and finally, potential confounders like ethnicity, *H. pylori* status (for gastric lesions), diet, NSAID use and lifestyle habits should be managed by subgroup analysis.

We may conclude that, although further research is needed, there are apparently consistent results (Table 6), both laboratory and observational, in *COX2* polymorphisms that may help to select a group of patients at higher risk of gastric cancer (−899G > C, −1329G > A, and *429T > C). This seems to be true among those with atrophic chronic gastritis or intestinal metaplasia (−899G > C and −1329G > A). Furthermore, in sporadic colon adenoma (V102V, V511A and *429T > G) and cancer (−1329G > A, −899G > C, V511A) *COX2* polymorphisms may help in defining a genetic profile of risk. If confirmed in future studies such as cohort studies or

Table 6 ***COX*** **polymorphisms expected impact based on bioinformatics predictive programs /functional studies (rows) and observed estimates (OR) on current study (columns)**

	Gastric carcinogenesis		Colorectal carcinogenesis	
COX polymorphisms	AIM	Gastric cancer	Colorectal adenoma	Colorectal cancer
Funcional				
COX1				
R8W			↔	↔
P17L			↔	
L237M	↔	↔	↔	[a]
COX2				
−1423A>G		↑↑↑		↔
−1329G>A	↔	↑↑↑	↔	↑↑↑
−899G>C	↔	↑↑↑	↔	↑↑↑[b]
IVS5−275T>G	↔	↔	↔	↔
*429T>C	↓↓	↓↓↓	↑↑↑[c]	↔
*1806A>G			↔	[a]
Nonfunctional				
COX2				
−1462_−1461delTG			↓	
−798A>G			↔	
V102V	↑	[a]	↓↓	↑
V511A			↓[d]	↓↓[d]
Unknown				
COX1				
IVS7+14delA	↔	↔		
IVS7−45T>C	↔	↔		
L15−L16del			↑↑	
V481I	↔	↑		↔
Q41Q	↔	↔		
G213G	↔	↔		
COX2				
−646C>T				↔
−196C>G				↔
−125T>G				↔
IVS7+111T>C		↔		
G587R	↓	↔		
*2291G>A				↑↑
*2430C>T		↑↑↑		

[a]Heterogeneity detected among studies.
[b]In Asiatic populations.
[c]Excluding the study by Gunter *et al.* [41] that has a C allele distribution very different from the other populations.
[d]In African–American individuals.
AIM, atrophy and/or intestinal metaplasia; CI, confidence interval; OR, odds ratio; ↔, no association; ↓, trend, ↓↓, strong trend, ↓↓↓, statistical association ($P<0.05$) for protection. Protection is defined as the 95% CI for OR<1; ↑, trend, ↑↑, strong trend, ↑↑↑, statistical association ($P<0.05$) for risk. Risk is defined as the 95% CI for OR>1.

else (e.g., cost-effectiveness analysis), these genetic profiles may enable clinicians to select individuals for early diagnosis strategies, diverse management schedules such as the follow-up of patients with intestinal metaplasia in the stomach or patients with earlier colonic adenoma (by anticipating follow-up examinations), or even to propose selective COX-2 inhibitors or nonspecific COX inhibitors in patients with precancerous lesions.

Supplementary data

Supplementary tables are available at *The European Journal of Gastroenterology & Hepatology* (online at *http://www.eurojgh.com*).

Acknowledgements

The authors would like to thank the Liga Portuguesa Contra o Cancro - Núcleo Regional do Norte. We would also like to acknowledge the Astra-Zeneca Foundation Research Grant 2004 and Gastroenterology Portuguese Society Research Grant of 2006 for their financial support.

Conflict of interest: none declared.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**:74–108.
2 Correa P. A human model of gastric carcinogenesis. *Cancer Res* 1988; **48**:3554–3560.
3 Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**:759–767.
4 Hamilton SR. The adenoma-adenocarcinoma sequence in the large bowel: variations on a theme. *J Cell Biochem Suppl* 1992; **16G**:41–46.
5 Yaghoobi M, Rakhshani N, Sadr F, Bijarchi R, Joshaghani Y, Mohammadkhani A, *et al*. Hereditary risk factors for the development of gastric cancer in younger patients. *BMC Gastroenterol* 2004; **4**:28.
6 Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; **12**:354–362.
7 American Cancer Society. *Colorectal Cancer Facts & Figures Special Edition 2005*. Atlanta: American Cancer Society; 2007.
8 Berlau J, Glei M, Pool-Zobel BL. Colon cancer risk factors from nutrition. *Anal Bioanal Chem* 2004; **378**:737–743.
9 Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860–867.
10 Marx J. Cancer research. Inflammation and cancer: the link grows stronger. *Science* 2004; **306**:966–968.
11 Macarthur M, Hold GL, El-Omar EM. Inflammation and Cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**:515–520.
12 Ulrich CM, Bigler J, Potter JD. Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. *Nat Rev Cancer* 2006; **6**:130–140.
13 Thun MJ, Henley SJ, Gansler T. Inflammation and cancer: an epidemiological perspective. *Novartis Found Symp* 2004; **256**:6–21.
14 Wang WH, Huang JQ, Zheng GF, Lam SK, Karlberg J, Wong BC. Non-steroidal anti-inflammatory drug use and the risk of gastric cancer: a systematic review and meta-analysis. *J Natl Cancer Inst* 2003; **95**: 1784–1791.
15 Jolly K, Cheng KK, Langman MJ. NSAIDs and gastrointestinal cancer prevention. *Drugs* 2002; **62**:945–956.
16 Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 1971; **231**:232–235.
17 Vane JR, Botting RM. Mechanism of action of nonsteroidal anti-inflammatory drugs. *Am J Med* 1998; **104**:2S–8S.
18 Chandrasekharan NV, Simmons DL. The cyclooxygenases. *Genome Biol* 2004; **5**:241.
19 DuBois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, *et al*. Cyclooxygenase in biology and disease. *FASEB J* 1998; **12**:1063–1073.
20 Bakhle YS. COX-2 and cancer: a new approach to an old problem. *Br J Pharmacol* 2001; **134**:1137–1150.
21 Cao Y, Prescott SM. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. *J Cell Physiol* 2002; **190**:279–286.
22 Fujimura T, Ohta T, Oyama K, Miyashita T, Miwa K. Role of cyclooxygenase-2 in the carcinogenesis of gastrointestinal tract cancers: a review and report of personal experience. *World J Gastroenterol* 2006; **12**:1336–1345.
23 van Rees BP, Ristimaki A. Cyclooxygenase-2 in carcinogenesis of the gastrointestinal tract. *Scand J Gastroenterol* 2001; **36**:897–903.
24 Wu AW, Gu J, Ji JF, Li ZF, Xu GW. Role of COX-2 in carcinogenesis of colorectal cancer and its relationship with tumor biological characteristics and patients' prognosis. *World J Gastroenterol* 2003; **9**:1990–1994.
25 Yu LZ, Gao HJ, Bai JF, Sun G, Zhao HL, Sun L, *et al*. Expression of COX-2 proteins in gastric mucosal lesions. *World J Gastroenterol* 2004; **10**:292–294.
26 Garcea G, Sharma RA, Dennison A, Steward WP, Gescher A, Berry DP. Molecular biomarkers of colorectal carcinogenesis and their role in surveillance and early intervention. *Eur J Cancer* 2003; **39**:1041–1052.
27 Zhang X, Miao X, Tan W, Ning B, Liu Z, Hong Y, *et al*. Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer. *Gastroenterology* 2005; **129**:565–576.
28 Guo Y, Zhang X, Tan W, Miao X, Sun T, Zhao D, *et al*. Platelet 12-Lipoxygenase Arg261Gln polymorphism: functional characterization and association with risk of esophageal squamous cell carcinoma in combination with COX-2 polymorphism. *Pharmacogenet Genomics* 2007; **17**:197–205.
29 Moons LM, Kuipers EJ, Rygiel AM, Groothuismink AZ, Geldof H, Bode WA, *et al*. COX-2 CA-haplotype is a risk factor for the development of esophageal adenocarcinoma. *Am J Gastroenterol* 2007; **102**:2373–2379.
30 Ferguson HR, Wild CP, Anderson LA, Murphy SJ, Johnston BT, Murray LJ, *et al*. Cyclooxygenase-2 and inducible nitric oxide synthase gene polymorphism and risk of reflux esophagitis, Barret's esophagus, and esophageal adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* 2008; **17**:727–731.
31 Zhang XM, Miao XP, Tan W, Sun T, Guo YL, Zhao D, *et al*. [Genetic polymorphisms in the promoter region of cyclooxygenase-2 and their association with risk of gastric cancer]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 2006; **28**:119–123.
32 Siezen CL, van Leeuwen AI, Kram NR, Luken ME, van Kranen HJ, Kampman E. Colorectal adenoma risk is modified by the interplay between polymorphisms in arachidonic acid pathway genes and fish consumption. *Carcinogenesis* 2005; **26**:449–457.
33 Zhang W, Gordon M, Press OA, Rhodes K, Vallböhmer D, Yang DY, *et al*. Cyclin D1 and epidermal growth factor polymorphisms associated with survival in patients with advanced colorectal cancer treated with Cetuximab. *Pharmacogenet Genomics* 2006; **16**:475–483.
34 Gordon MA, Gil J, Lu B, Zhang W, Yang D, Yun J, *et al*. Genomic profiling associated with recurrence in patients with rectal cancer treated with chemoradiation. *Pharmacogenomics* 2006; **7**:67–88.
35 Pereira C, Sousa H, Ferreira P, Fragoso M, Moreira-Dias L, Lopes C, *et al*. –765G>C COX-2 polymorphism may be a susceptibility marker for gastric adenocarcinoma in patients with atrophy or intestinal metaplasia. *World J Gastroenterol* 2006; **12**:5473–5478.
36 Liu F, Pan K, Zhang X, Zhang Y, Zhang L, Ma J, *et al*. Genetic variants in cyclooxygenase-2: Expression and risk of gastric cancer and its precursors in a Chinese population. *Gastroenterology* 2006; **130**:1975–1984.
37 Ulrich CM, Bigler J, Sparks R, Whitton J, Sibert JG, Goode EL, *et al*. Polymorphisms in PTGS1 (=COX-1) and risk of colorectal polyps. *Cancer Epidemiol Biomarkers Prev* 2004; **13**:889–893.
38 Ulrich CM, Whitton J, Yu JH, Sibert J, Sparks R, Potter JD, *et al*. PTGS2 (COX-2) –765G>C promoter variant reduces risk of colorectal adenoma among nonusers of nonsteroidal anti-inflammatory drugs. *Cancer Epidemiol Biomarkers Prev* 2005; **14**:616–619.
39 Ali IU, Luke BT, Dean M, Greenwald P. Allellic variants in regulatory regions of cyclooxygenase-2: association with advanced colorectal adenoma. *Br J Cancer* 2005; **93**:953–959.
40 Siezen CL, Tijhuis MJ, Kram NR, van Soest EM, de Jong DJ, Fodde R, *et al*. Protective effect of nonsteroidal anti-inflammatory drugs on colorectal adenomas is modified by a polymorphism in peroxisome proliferator-activated receptor delta. *Pharmacogenet Genomics* 2006; **16**:43–50.
41 Gunter MJ, Canzian F, Landi S, Chanock SJ, Sinha R, Rothman N. Inflammation-related gene polymorphisms and colorectal adenoma. *Cancer Epidemiol Biomarkers Prev* 2006; **15**:1126–1131.

42 Lin HJ, Lakkides KM, Keku TO, Reddy ST, Louie AD, Kau IH, *et al*. Prostaglandin H synthase 2 variant (Val511Ala) in African Americans may reduce the risk for colorectal neoplasia. *Cancer Epidemiol Biomarkers Prev* 2002; **11**:1305–1315.
43 Hamajima N, Takezaki T, Matsuo K, Saito T, Inoue M, Hirai T, *et al*. Genotype Frequencies of Cyclooxygenease 2 (COX2) Rare Polymorphisms for Japanese with and without Colorectal Cancer. *Asian Pac J Cancer Prev* 2001; **2**:57–62.
44 Tan W, Wu J, Zhang X, Guo Y, Liu J, Sun T, *et al*. Associations of functional polymorphisms in cyclooxygenase-2 and platelet 12-lipoxygenase with risk of occurrence and advanced disease status of colorectal cancer. *Carcinogenesis* 2007; **28**:1197–1201.
45 Cox DG, Pontes C, Guino E, Navarro M, Osorio A, Canzian F, *et al*. Polymorphisms in prostaglandin synthase 2/cyclooxygenase 2 (PTGS2/COX2) and risk of colorectal cancer. *Br J Cancer* 2004; **91**: 339–343.
46 Landi S, Gemignani F, Bottari F, Gioia-Patricola L, Guino E, Cambray M, *et al*. Polymorphisms within inflammatory genes and colorectal cancer. *J Negat Results Biomed* 2006; **5**:15.
47 Goodman JE, Bowman ED, Chanock SJ, Albert AJ, Harris CC. Arachidonate lipoxygenase (ALOX) and cyclooxygenase (COX) polymorphisms and colon cancer risk. *Carcinogenesis* 2004; **25**:2467–2472.
48 Siezen CL, Bueno-de-Mesquita HB, Peeters PH, Kram NR, van DM, van Kranen HJ. Polymorphisms in the genes involved in the arachidonic acid-pathway, fish consumption and the risk of colorectal cancer. *Int J Cancer* 2006; **119**:297–303.
49 Koh WP, Yuan JM, van den BD, Lee HP, Yu MC. Interaction between cyclooxygenase-2 gene polymorphism and dietary n-6 polyunsaturated fatty acids on colon cancer risk: the Singapore Chinese Health Study. *Br J Cancer* 2004; **90**:1760–1764.
50 Sansbury LB, Millikan RC, Schroeder JC, North KE, Moorman PG, Keku TO, *et al*. COX-2 polymorphism, use of nonsteroidal anti-inflammatory drugs, and risk of colon cancer in African Americans (United States). *Cancer Causes Control* 2006; **17**:257–266.
51 Xing LL, Wang ZN, Jiang L, Zhang Y, Xu YY, Luo Y, *et al*. Cyclooxygenase 2 polymorphism and colorectal cancer: –765G>C variant modifies risk associated with smoking and body mass index. *World J Gastroenterol* 2008; **14**:1785–1789.
52 Saxena A, Prasad KN, Ghoshal UC, Bhagat MR, Krishnani N, Husain N. Polymorphism of –765G>C *COX-2* is a risk factor for gastric adenocarcinoma and peptic ulcer disease in addition to *H. pylori* infection: a study from northern India. *World J Gastroenterol* 2008; **14**:1498–1503.
53 Canzian F, Franceschi S, Plummer M, van Doorn LJ, Lu Y, Gioia-Patricola L, *et al*. Genetic polymorphisms in mediators of inflammation and gastric precancerous lesions. *Eur J Cancer Prev* 2008; **17**:178–183.
54 Ueda N, Maehara Y, Tajima O, Tabata S, Wakabashi K, Kono S. Genetic polymorphisms of *cyclooxygenase-2* and colorectal adenoma risk: the Self Defense Forces Health Study. *Cancer Sci* 2008; **99**:576–781.
55 Hou L, Grillo P, Zhu ZZ, Lissowska J Yeager M, Zatonski W, *et al*. *COX1* and *COX2* polymorphisms and gastric cancer risk in a Polish population. *Anticancer Res* 2007; **27**:4243–4247.
56 Poole EM, Bigler J, Whitton J, Sibert JG, Kulmacz RJ, Potter JD, *et al*. Genetic variability in prostaglandin synthesis, fish intake and risk of colorectal polyps. *Carcinogenesis* 2007; **28**:1259–1263.
57 Sitarz R, Leguit RJ, de Leng WW, Polak M, Morsink FM, Bakker O, *et al*. The *COX-2* promoter polymorphism in –765G>C is associated with early-onset, conventional and stump gastric cancers. *Mod Pathol* 2008; **21**:685–690.
58 Hubner RA, Muir KR, Liu JF, Logan RF, Grainge MJ, Houlston RS, *et al*. Polymorphisms in PTGS1, PTGS2 and IL-10 do not influence colorectal recurrence in the context of a randomized aspirin intervention trial. *Int J Cancer* 2007; **121**:2001–2004.
59 Saukken K, Nieminen O, van Rees B, Vilkki S, Härkönem M, Juhola M, *et al*. Expression of cyclooxygenase-2 in dysplasia of the stomach and in intestinal-type gastric adenocarcinoma. *Clin Cancer Res* 2001; **7**:1923–1931.
60 Thakkinstian A, McEvoy M, Minelli C, Gibson P, Hancox B, Duffy D, *et al*. Systematic review and meta-analysis of the association between (beta)2-adrenoceptor polymorphisms and asthma: a HuGE review. *Am J Epidemiol* 2005; **162**:201–211.
61 STROBE statement: Checklist of essential items Version 3 (Sept 2005).
62 den Dunnen JT, Antonarakis SE. Nomenclature for the description of human sequence variations. *Hum Genet* 2001; **109**:121–124.
63 Review Manager (RevMan) [computer program]. Version 4.2. for Windows. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2003.
64 DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**:177–188.
65 Zintzaras E, Ioannidis JP. Heterogeneity testing in meta-analysis of genome searches. *Genet Epidemiol* 2005; **28**:123–137.
66 Sterne JA, Egger M, Smith GD. Systematic reviews in health care: Investigating and dealing with publication and other biases in meta-analysis. *Br Med J* 2001; **323**:101–105.
67 Knudsen LE, Loft SH, Autrup H. Risk assessment: the importance of genetic polymorphisms in man. *Mutat Res* 2001; **482**:83–88.
68 Ulrich CM, Bigler J, Sibert J, Greene EA, Sparks R, Carlson CS, *et al*. Cyclooxygenase 1 (COX1) polymorphisms in African-American and Caucasian populations. *Hum Mutat* 2002; **20**:409–410.
69 Shi J, Misso NL, Duffy DL, Bradley B, Beard R, Thompson PJ, *et al*. Cyclooxygenase-1 gene polymorphisms in patients with different asthma phenotypes and atopy. *Eur Respir J* 2005; **26**:249–256.
70 Picot D, Loll PJ, Garavito RM. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature* 1994; **367**:243–249.
71 Lee CR, North KE, Bray MS, Couper DJ, Heiss G, Zeldin DC. Cyclooxygenase polymorphisms and risk of cardiovascular events: the atherosclerosis risk in communities (ARIC) study. *Clin Pharmacol Ther* 2008; **83**:52–60.
72 Ahmed FE. Gene-gene, gene environment & multiple interactions in colorectal cancer. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2006; **24**:1–101.
73 Papafili A, Hill MR, Brull DJ, McAnulty RJ, Marshall RP, Humphries SE, *et al*. Common promoter variant in cyclooxygenase-2 represses gene expression: evidence of role in acute-phase inflammatory response. *Arterioscler Thromb Vasc Biol* 2002; **22**:1631–1636.
74 Szczeklik W, Sanak M, Szczeklik A. Functional effects and gender association of COX-2 gene polymorphism G-765C in bronchial asthma. *J Allergy Clin Immunol* 2004; **114**:248–253.
75 Kanaoka S, Takai T, Yoshida K. Cyclooxygenase-2 and tumor biology. *Adv Clin Chem* 2007; **43**:59–78.
76 Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresalier R, *et al*. A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med* 2003; **348**:891–899.
77 Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, *et al*. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med* 2003; **348**:883–890.
78 Chan AT, Giovannucci EL, Meyerhardt JA, Schernhammer ES, Curhan GC, Fuchs CS. Long-term use of aspirin and nonsteroidal anti-inflammatory drugs and risk of colorectal cancer. *J Am Med Assoc* 2005; **294**: 914–923.
79 Garcia-Rodriguez LA, Huerta-Alvarez C. Reduced risk of colorectal cancer among long-term users of aspirin and nonaspirin nonsteroidal antiinflammatory drugs. *Epidemiology* 2001; **12**:88–93.
80 Dixon DA. Regulation of COX-2 expression in human cancers. *Prog Exp Tumor Res* 2003; **37**:52–71.
81 Dixon DA, Kaplan CD, McIntyre TM, Zimmerman GA, Prescott SM. Post-transcriptional control of cyclooxygenase-2 gene expression. The role of the 3′-untranslated region. *J Biol Chem* 2000; **275**:11750–11757.
82 Campa D, Zienolddiny S, Maggini V, Skaug V, Haugen A. Association of a common polymorphism in the cyclooxygenase 2 gene with risk of non-small cell lung cancer. *Carcinogenesis* 2004; **25**:229–235.
83 Attia J, Thakkinstian A, D'Este C. Meta-analyses of molecular association studies: methodologic lessons for genetic epidemiology. *J Clin Epidemiol* 2003; **56**:297–303.

Table A. Scale for Quality Assessment

Criteria	Score 0 to 50
Title and Abstract: Is the article identified as an observational study (case-control or cohort study) in the title or abstract? (1 - yes; 0 - no)	□ 0 □ 1
Title and Abstract: Is the abstract informative and structured? (1 - yes; 0 - no)	□ 0 □ 1
Introduction: Does the introduction explain scientific background and the rationale for the research being reported? (1 - yes; 0 - no)	□ 0 □ 1
Introduction: Does the introduction include a clear statement of objectives? (1 - yes; 0 - no)	□ 0 □ 1
Methods / Study design: Does the article present all key elements of study design (including designation and correctness of study designation)? (2 - yes; 0 - no)	□ 0 □ 2
Methods / Setting: Does the article describe setting (includes locations and dates for data collection)? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are inclusion and exclusion for cases clear stated? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are methods of selection described? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are clear diagnostic criteria stated for cases (in case-controls)? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are cases based on histological confirmation (ascertainment of cases)? (1 - yes; 0 - no)	□ 0 □ 1
Bias: Are cases adequately representative (selection bias)? (2 – From cancer registries; 1 – From clinical databases; 0 – w/o clearly defined sampling frame)	□ 0 □ 1 □ 2
Participants: Is the rationale for controls stated? (1 - yes; 0 - no)	□ 0 □ 1
Bias: Are sources of controls adequate? (3- population or neighbour-based; 2- blood donors; 1- hospital based or healthy not-stated; 0- not described)	□ 0 □ 1 □ 2 □ 3
Participants: Are matching criteria for controls stated? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are controls histological based (ascertainment of cases)? (2 - yes; 0 - no)	□ 0 □ 2
Variables of interest: Are all outcomes variables adequately described? (1 - yes; 0 - no)	□ 0 □ 1
Bias: Were potential confounders or effect modifiers adequately taken care? (2 - yes; 0 - no)	□ 0 □ 2
Measurements: Are details of methods of measurements given? (1 - yes; 0 - no)	□ 0 □ 1
Measurements: Are measurements performed equally between cases and controls? (2- yes; 0 - no)	□ 0 □ 2
Bias: Are measurements of exposure made blinded/independently measured to group (in case-controls) or definition of outcome blinded to exposure (in cohorts)? (2 - yes; 0 - no)	□ 0 □ 2
Hardy-Weinberg equilibrium: Was HWE assessed? (1 - yes; 0 - no)	□ 0 □ 1
Hardy-Weinberg equilibrium: Were control populations in HWE? (1 - yes; 0 - no)	□ 0 □ 1
Sample size: Are sample size estimates described? (1 - yes; 0 - no)	□ 0 □ 1
Statistical methods: Were methods used to assess association between exposure (polymorphisms) and outcome (cancer) adequate? (1 - yes; 0 - no)	□ 0 □ 1
Statistical methods: was management of confounders taken care (subgroups analysis)? (1 - yes; 0 - no)	□ 0 □ 1
Statistical methods: Was missing data managed in case-controls? (1 - yes; 0 - no)	□ 0 □ 1
Or	
Bias: In cohort studies, was the follow-up more than 80%? (1 - yes; 0 - no)	□ 0 □ 1
Funding and disclosure statement: Are funding and/or disclosure clearly stated?	□ 0 □ 1
Participants: Does the manuscript reports adequately the numbers of individuals at each stage of study (eligible, participating and analysed)? (3 - yes; 0 - no)	□ 0 □ 3
Participants: Are reasons of non-participation presented? (1 - yes; 0 - no)	□ 0 □ 1

Table A. Scale for Quality Assessment

Criteria	Score 0 to 50
Participants: Does a flow diagram presented? (1 - yes; 0 - no)	□ 0 □ 1
Internal validity: Are selected and participants similar in important variables? (2 - yes; 0 - no)	□ 0 □ 2
Descriptive data: Are characteristics of participants described (eg, demographic, clinical, social)? (1 - yes; 0 - no)	□ 0 □ 1
Outcome data: Does the manuscript describe adequately the numbers of outcomes events in exposed, or, of exposition in cases and controls? (1 - yes; 0 - no)	□ 0 □ 1
Main results: Are measures of association presented with precision estimates (95% CI)? (1 - yes; 0 - no)	□ 0 □ 1
Main results: Are measures of association adjusted to confounders? (2 - yes; 0 - no)	□ 0 □ 2
Discussion: Does the discussion include a summary of main results? (1 - yes; 0 - no)	□ 0 □ 1
Limitations: Are limitations discussed? (1 - yes; 0 - no)	□ 0 □ 1
Generability and interpretation: Does the manuscript discuss generability? (1 - yes; 0 - no)	□ 0 □ 1

Table B. Polymorphisms characterization

Polymorphism § (other names Ω)	dbSNP # (rs number)	Nucleotide position Φ	Nucleotide alteration ¥	amino acid (a.a.) alteration	Biological plausibility	Function
COX-1						
Intronic region						
IVS7+14delA	rs3215925	11707	Deletion of A (intron 7)	-	Unknown	Unknown
IVS7-45T>C	rs3842798	13410	g.12380T>C (intron 7)	-	Unknown	Unknown
Coding region						
L15_L16del	(1)	-	-	Deletion of two Leucines (L) in the COX1 signal peptide region	Probably has little impact in protein location[37]†	Unknown
p.R8W	rs1236913	1149	g.116C>T (exon 2)	Arginine (R)-to-Tryptophan (W) at a.a.8	Probably affect protein function[68]†; may alter the binding of putative splicing factors (SR proteins)[69]†	Normal expression[71]‡
p.P17L	rs3842787	1176	g.144C>T (exon 2)	Proline (P)-to-Leucine (L) at a.a.17	Probably affect protein function[68]†; may alter the binding of putative splicing factors (SR proteins)[69]†	Normal expression[71]‡
p.Q41Q	rs3842788	7873	g.6843A>G (exon 3)	Glutamine (Q)-to-Glutamine at a.a. 41	Silent polymorphism	Unknown
p.G213G	rs5788	11459	g.10429C>A (exon 6)	Glycine (G)-to-Glycine at a.a.213	Silent polymorphism	Unknown
p.L237M	rs5789	11640	g.10608C>A (exon 7)	Leucine (L)-to-Methionine (M) at a.a.237	Located near the COX-1 dimer interface connecting two identical monomeric subunits (new hydrogen bond);[68,70] Probably affect protein function[68]†	Decreased activity[71]‡
p.V481I (V444I)	rs5794	20287	g.19255G>A (exon 10)	Valine (V)-to-Isoleucine (I) at a.a.481	Unknown	Unknown
COX-2						
Promoter region						
-1462_-1461delTG (-663GT/(GT))	rs689464	362	Deletion of TG	-	Not in transcription factors binding sites[39]†	Unknown
-1423A>G (-1290A>G, 401)	rs689465	401	g.-1423A>G	-	Unknown	Unknown
-1329G>A (-1195G>A)	rs689466	496	g. -1329G>A	-	Creation of a C-MYB binding site[27]†	Higher transcription activity of the *COX-2* gene[27]‡

Polymorphism § (other names Ω)	dbSNP # (rs number)	Nucleotide position Φ	Nucleotide alteration ¥	amino acid (a.a.) alteration	Biological plausibility	Function
-899G>C (-765G>C, 926)	rs20417	926	g.-899G>C	-	Disruption of Sp1 binding site[73] OR possible creation of E2F binding site that may enhance COX-2 expression[74]†	30% reduction in the promoter activity[73]‡; 10-fold increased production of PGE_2[74]
-798A>G	(2)	-	A>G	-	Not in transcription factors binding sites[39]†	Unknown
-646C>T	rs20420	1179	g-646C>G	-	Unknown	Unknown
5' UTR (exon 1)						
-196C>G (1629)	rs20424	1629	g.-196C>G	-	Unknown	Unknown
-125T>G (10T>C)	rs5271	1700	g.-125T>G	-	Unknown	Unknown
Intronic region						
IVS5-275T>G (5229, 5209)	rs20432	5209	g.3385T>G (intron 5)	-	Unknown	Unknown
IVS7+111T>C	rs4648276	6043	g.4219T>C (intron 7)	-	Unknown	Unknown
Coding region						
p.V102V (Ex3-8G>C, 3050)	rs5277	3050	g.1226G>C (exon 3)	Valine (V)-to-Valine (V) at a.a.102	Silent polymorphism	Unknown
p.V511A	rs5273	7763	g.5939T>C (exon 10)	Valine (V)-to-Alanine (A) change at a.a.511	Lies inside a tighly packed hydrophobic pocket adjacent to the cyclooxygenase active site[42]	No alteration in enzyme kinetic parameters (Vmáx and Km) or the stability for the utilization of arachidonic acid[42]‡
p.G587R (Gly587Arg)	rs3218625	7990	g.6166G>A (exon 10)	Glycine (G)-to-Arginine (R) change at a.a.587	Unknown	Unknown
3'UTR (exon 10)						
***429T>C** (2242T>C; 8494T>C; 8473 Ex10+837C>T)	rs5275	8473	g.6649T>C	-	SNP located in the AU-rich region that mediates transcript degradation that might have a transcript-stabilising function[39,82]	Unknown
***1806A>G** (3618A>G; 9850)	rs4648298	9850	g.8026A>G	-	May have some effect on the addition of some poly-A tail to the mRNA, or can cause a later poly-A site to be used, creating a longer, and possibly more stable species of mRNA[45]	Unknown
***2291G>A** (10335)	rs689469	10335	g.8511G>A	-	Downstream of the last polyadenylation site[45]	Unknown
***2430C>T**	rs689470	10474	g.8650C>T	-	Unknown	Unknown

CHAPTER IB: *COX-2* POLYMORPHISMS AND COLORECTAL CANCER RISK: A STRATEGY FOR CHEMOPREVENTION

COX-2 polymorphisms and colorectal cancer risk: a strategy for chemoprevention

Carina Pereira[a,c], Pedro Pimentel-Nunes[b,d], Catarina Brandão[b], Luís Moreira-Dias[b], Rui Medeiros[a,f] and Mário Dinis-Ribeiro[b,e]

Objective ***COX-2*, the inducible isoenzyme, was found to be overexpressed in approximately 85% of colorectal adenocarcinomas, contributing to key steps in tumor development. *COX-2* polymorphisms that might modify the levels of protein expression would be anticipated to have a substantial influence on disease phenotype. Therefore, we sought to understand the role of three *COX-2* polymorphisms (−1195A>G, −765G>C, and 8473T>C) in colorectal cancer (CRC) onset.**

Material and methods **We conducted a hospital-based case–control study involving 117 consecutively enrolled CRC patients and 256 healthy individuals without any clinical evidence of cancer. The *COX-2* polymorphisms' genotypes were characterized by PCR-restriction fragment length polymorphism or real-time PCR techniques.**

Results **The −1195A>G polymorphism was associated with a 1.73-fold increased predisposition to CRC onset. In a stratified analysis, men and ever-smokers carrying −1195G allele (AG+GG) had an increased risk for CRC development (odds ratio: 2.58; 95% confidence intraval: 1.29–5.15 and odds ratio: 10.3; 95% confidence intraval: 3.37–31.2, respectively). More interestingly, men ever-smokers carrying −1195G allele appeared to have a nine-fold increased risk for CRC onset (95% CI: 2.94–27.6). No difference in the genotype's distribution was noticed between cases and controls for the remaining two polymorphisms.**

Conclusion **The −1195A>G *COX-2* polymorphism seems to modulate the genetic susceptibility for CRC onset, especially in men ever-smokers. This genetically based higher-risk group definition may help shift the balance between risk and benefits for the use of *COX-2* inhibitors in chemoprevention that is currently hampered by the adverse gastrointestinal and cardiovascular side-effects. *Eur J Gastroenterol Hepatol* 00:000–000 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

European Journal of Gastroenterology & Hepatology 2010, **00**:000–000

Keywords: colorectal cancer, cyclooxygenase-2, genetic susceptibility, polymorphisms

[a]Molecular Oncology Group, [b]Gastroenterology Department, Portuguese Institute of Oncology, [c]Liga Portuguesa Contra o Cancro, Núcleo Regional do Norte, [d]Physiology Department, [e]CINTESIS/Department of Biostatistics and Medical Informatics, Faculty of Medicine, University of Porto and [f]ICBAS, Abel Salazar Institute for the Biomedical Sciences, University of Porto, Port, Portugal

Corresponence to Dr Carina Pereira, MSc, Grupo de Oncologia Molecular, Instituto Português de Oncologia de Francisco Gentil – EPE Rua Dr. António Bernardino Almeida, Porto 4200-072, Portugal
Tel: +351 22 508 4000 x5413; fax: +351 22 508 4001;
e-mail: anacmpereira@gmail.com

Received 22 July 2009 **Accepted** 8 November 2009

Introduction

In developed countries, colorectal cancer (CRC) is the second most widespread malignancy with a lifetime risk of 5%, mainly imputed to the 'westernized lifestyle' [1–3].

Traditional nonsteroidal anti-inflammatory drugs, such as aspirin, are thought to exert their chemopreventive actions mainly by targeting cyclooxygenase (COX) enzymes [4]. COX-1 is constitutively expressed in a wide range of organs and responsible for tissue homeostasis. Although normally undetectable in physiological conditions, COX-2 is induced under inflammatory and tumor promotion stimuli, being overexpressed in approximately 50% of adenomas and 85% of colon adenocarcinomas [5].

In an original observational study, Chan *et al.* [6] observed that aspirin's preventive role was effective only in the subgroup of colon cancers overexpressing COX-2 in a dose and treatment duration dependent manner. Hence, the future challenge lies in the identification of individuals who will express higher levels of COX-2, probably through the interaction between the genetic background and environmental exposure [7].

The involvement of *COX-2* genetic variations in colorectal tumor development has been proposed [8]. The −1195A > G (rs689466) and −765G > C (rs20417) polymorphisms, identified in gene's promoter region, are expected to modulate COX-2 expression by altering the recognition binding site for specific nuclear proteins, thus influencing the genetic susceptibility for CRC onset [9,10]. In contrast, the 8473T > C (rs5275) polymorphism in an AU-rich elements region (3′UTR) might contribute to cancer development by influencing COX-2 mRNA stability [11–13]. In this study, we sought to evaluate the influence of COX-2 functional polymorphisms in the development of CRC. We also aimed to investigate possible interactions between these genetic variations and environmental factors in CRC onset.

Supplemental digital content is available for this directly from the author.

0954-691X © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/MEG.0b013e3283352cbb

Material and methods

Study population

This hospital-based case–control study included 373 participants: 117 histologically confirmed CRC patients and 256 cancer-free controls from the northern region of Portugal recruited at the Portuguese Institute of Oncology, Porto. The method of sampling for cases and controls can be observed in Fig. 1. Eligible cases included all patients with a newly diagnosed CRC between January 2002 and September 2007 selected from a colonoscopy database from the Gastroenterology department, aged 50–75 years, without a previous history of inflammatory bowel diseases or hereditary syndromes and who were scheduled for a follow-up observation at our institution between March and May 2008 ($n = 384$). Controls were healthy individuals without any clinical evidence of CRC selected from a DNA database of more than 1000 blood donors consecutively recruited at our institute between July 2005 and October 2007. All the individuals between 50 and 75 years of age were included ($n = 307$).

Overall, the participation rate for the interviewed individuals meeting the cases' inclusion criteria ($n = 166$) was 90% ($n = 150$). We were unable to obtain blood samples from all the included cases by the time frame of this study, but the distribution of known risk factors for CRC [age, sex, body mass index (BMI) and smoking behavior] did not deviate between the genotyped group ($n = 117$) and the included population. In the control group, DNA samples were available only from 256 (83%) participants to allow the genotype characterization of all *COX-2* polymorphisms.

Written informed consent was obtained from all participants before their inclusion in the study, according to the Declaration of Helsinki. Furthermore, this research was approved by the Ethics Committee of the Portuguese Institute of Oncology, Porto. The methods for data collection and variables definition can be seen in Supplementary data 1.

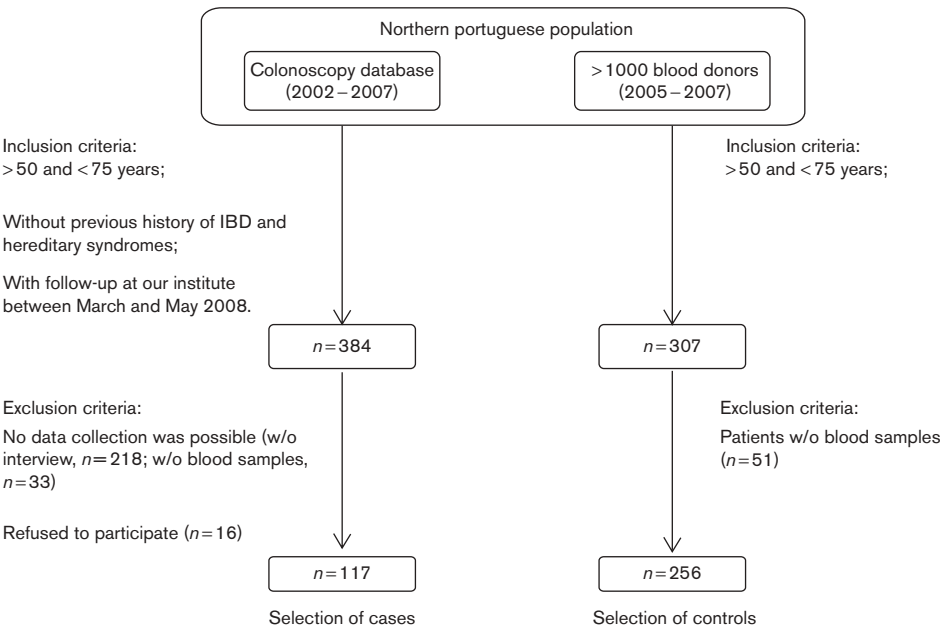
Sample DNA extraction

Blood samples were collected with a standard venipuncture technique using EDTA-containing tubes. Genomic DNA was extracted from peripheral blood leukocytes, using the QIAamp DNA Blood Mini Kit (Qiagen, Madrid, Spain) following the manufacturer's instructions.

COX-2 polymorphisms genotyping

PCR-based restriction fragment length polymorphism technique was used to characterize the different genotypes of −1195A > G and −765G > C *COX-2* polymorphisms as described earlier [10,14]. Briefly, the PCRs were performed using a specific pair of primers (Metabion, Martinsried, Deutschland) to amplify the DNA fragments containing the −1195A > G (F5′-CCCTGAGA CACTACCCATGAT-3′ and R5′-GCCCTTCAT AGGAGA TACTGG-3′) and −765G > C (F5′-ATTCTGGCCATC GCCGCTTC-3′ and R5′-CTCCTTGTTTCTTGGAAA GAGACG-3′) *COX-2* polymorphisms. Afterwards, the amplified fragments were digested with 1U of *PvuII* and *Bsh1236I* restriction endonucleases (Fermentas, Vilnius, Lithuania) allowing the genotype characterization of −1195A > G (AA: 273 bp; GG:220 + 53 bp; AG:273 +

Fig. 1



Methods of sampling for cases and controls. IBD, inflammatory bowel disease; w/o, without.

220 + 53 bp) and −765G > C (GG:134 + 23 bp; CC: 157 bp: GC:157 + 134 + 23 bp) *COX-2* polymorphisms, correspondingly.

For quality control, (i) negative controls were included in every PCR reaction; (ii) the genotype interpretation was independently performed by two experienced researchers. There was no discrepancy between results; (iii) a second PCR-restriction fragment length polymorphism analysis was randomly repeated in 10% of all samples for each polymorphism. The 8473T > C polymorphism was genotyped through an allelic discrimination analysis on a ABI Prism 7300 Real-Time PCR System (Applied Biosystems, Foster City, California, USA) using the following validated TaqMan SNP genotyping assay (Applied Biosystems): C___7550203_10. Cases with undetermined genotype even after a second round were excluded. Likewise, in each 96-well plate, negative controls were included and 10% of genotyped samples were randomly selected for a second analysis.

Statistical analysis

The Hardy–Weinberg equilibrium was tested by the Pearson's goodness-of-fit test to compare the observed versus the expected genotype frequencies.

Data analysis was performed using the computer software *Statistical Package for Social Sciences-SPSS* (SPSS Inc., Chicago, Illinois, USA) for Windows (version 15.0). Chi-square analysis was used to compare categorical variables, using a 5% level of significance. Statistical differences between mean values were evaluated by applying the Mann–Whitney test.

Multivariate logistic regression analysis was used to estimate odds ratio (OR) and its 95% confidence interval (CI) as a measure of the association between variant allele carriers and the risk for the development of CRC. The potential confounding variables such as age, sex, BMI, and smoking habits were addressed either by being included as covariates in the multivariate analysis and/or through data stratification.

Homozygotes for the allele with the highest frequency were used as the reference group for each OR estimation. Variant allele carriers were defined as the heterozygous and minor allele homozygous genotype carriers pooled together (dominant model).

Results

Participants' description

The characteristics of the study population are summarized in Table 1. Cases were significantly older than controls with a median age of 61 years (50–75) [vs. 55 years in controls (50–65), $P < 0.001$].

There were no significant differences in the distribution of sex, BMI, and smoking habits between both groups. Males represented 63% of either population ($P = 0.995$), and 82% of cases were overweight (vs.76% in controls, $P = 0.243$).

Table 1 Description of participants

	Cases ($n=117$)	Controls ($n=256$)	*P* value
Demographics			
Age (years)			
Mean (SD)	62 (4.1)	56 (6.9)	
Median (min–max)	61 (50–65)	55 (50–75)	<0.001[d]
Sex, *n* (%)			
Male	74 (63.2)	162 (63.3)	0.995
Female	43 (36.8)	94 (36.7)	
Lifestyle behaviors[b]			
BMI (kg/m^2)			
Mean (SD)	28 (4.0)	28 (3.6)	
Median (min–max)	28 (20–43)	27 (21–40)	0.643[d]
BMI category, *n* (%)[a]			
<25	21 (18.3)	40 (24.1)	0.243
≥ 25	94 (81.7)	126 (75.9)	
Smoking status, *n* (%)			
Never-smokers	68 (58.1)	104 (65.4)	0.217
Ever-smokers	49 (41.9)	55 (34.6)	
UPY	37 (31–43)	23 (18–28)	0.002
Tumor characteristics[c]			
Tumor location, *n* (%)			
Recto	44 (37.6)	–	
Colon	73 (62.4)	–	
Stage, *n* (%)			
I–II	57 (48.7)	–	
III–IV	60 (51.3)	–	
Grade, *n* (%)			
Low/Intermediate	88 (75.2)	–	
High	5 (4.3)	–	
Undefined	24 (20.5)	–	
Synchronous tumors, *n* (%)			
Yes	7 (6.0)	–	
No	110 (94.0)	–	

BMI, body mass index; UPY, unit packs (day) × years.
[a]Categorization based on the cutoff defined by WHO for overweight people [15].
[b]The numbers may not add up, as we were unable to gather this information for all participants, namely in the control group.
[c]For synchronous tumors the most advanced lesion was the one considered in tumors' characterization.
[d]*P* value was estimated using the non-parametric Mann–Whitney test.

Genotype frequencies and risk estimates

Genotype frequency of each *COX-2* polymorphism according to disease status is displayed in Table 2. The genotypic distribution of all three single nucleotide polymorphisms (SNPs) in the control group was in agreement with the Hardy–Weinberg equilibrium principles ($P \geq 0.05$) and the frequencies for the −1195G, −765C, and 8473C alleles were 16.6, 18.9, and 32.4%, respectively. No significant differences in genotype distribution were noticed for the *COX-2* genetic variations addressed, although, the heterozygous −1195AG genotype was over-represented in cases (36.8 vs. 28.5%, $P = 0.094$). Furthermore, the G allele carriers showed an increased risk trend for CRC onset (OR: 1.50; 95% CI: 0.955–2.371, $P = 0.078$) that reaches significance level in a multivariate analysis addressing potential confounders for CRC (age, sex, BMI and smoking habits) (OR: 1.74; 95% CI: 1.011–2.975, $P = 0.045$).

Table 2 Genotype frequencies among cases and controls and univariate and multivariate OR (95% CI) estimation on the role of *COX-2* polymorphisms in colorectal cancer onset

	Univariate analysis					Multivariate analysis			
Polymorphism	Cases	Controls	OR	95% CI	*P* value	*N*[a]	aOR	95% CI	*P* value
−1195A>G									
AA	70 (59.8)	177 (69.1)	1.00	Reference	–		1.00	Reference	–
AG	43 (36.8)	73 (28.5)	1.489	0.933–2.377	0.094	267	1.673	0.964-2.903	0.067
GG	4 (3.4)	6 (2.3)	1.686	0.462–6.155	0.425	187	3.170	0.468-21.48	0.237
G carriers	47 (40.2)	79 (30.9)	1.504	0.955–2.371	0.078	272	1.735	1.011-2.975	0.045
−765G>C									
GG	77 (65.8)	166 (64.8)	1.00	Reference	–		1.00	Reference	–
GC	38 (32.5)	83 (32.4)	0.987	0.617–1.578	0.956	265	0.862	0.500-1.486	0.592
CC	2 (1.7)	7 (2.7)	0.616	0.125–3.034	0.548	179	0.359	0.060-2.161	0.263
C carriers	40 (34.2)	90 (35.2)	0.958	0.605–1.518	0.856	272	0.812	0.477-1.383	0.444
8473T>C									
TT	54 (47.0)	118 (46.1)	1.00	Reference	–		1.00	Reference	–
TC	51 (44.3)	114 (44.5)	0.978	0.616–1.550	0.923	248	0.869	0.510-1.480	0.604
CC	10 (8.7)	24 (9.4)	0.910	0.407–2.036	0.819	147	0.858	0.314-2.347	0.766
C carriers	61 (53.0)	138 (53.9)	0.966	0.621–1.501	0.878	270	0.874	0.525-1.457	0.606

aOR, adjusted odds ratio for age, sex, body mass index, and smoking status; CI, confidence interval; OR, odds ratio.
[a]Total number of participants included in the multivariate analysis.

Table 3. aOR (95% CI), under the dominant model, for the influence of *COX-2* polymorphims in colorectal cancer onset stratified for age, sex, BMI, and smoking status

	COX-2 polymorphisms									
	−1195A>G (G carriers vs. AA)				−765G>C (C carriers vs. GG)			8473T>C (C carriers vs. TT)		
Stratification	*N*	aOR	95% CI	*P* value	aOR	95% CI	*P* value	aOR	95% CI	*P* value
Age (years)[a]										
<57	125	1.423	0.604–3.353	0.419	1.577	0.688–3.617	0.282	1.336	0.577–3.093	0.499
≥ 57	147	1.933	0.946-3.952	0.071	0.552	0.270–1.131	0.104	0.736	0.376–1.438	0.369
Sex										
Female	97	0.632	0.243–1.643	0.346	0.923	0.345–2.467	0.872	0.623	0.252–1.541	0.306
Male	175	2.579	1.290–5.154	0.007	0.854	0.441–1.651	0.638	1.060	0.559–2.011	0.585
BMI (kg/m^2)										
<25	57	2.479	0.686–8.956	0.166	0.366	0.106–1.270	0.113	0.549	0.160–1.884	0.341
≥ 25	215	1.608	0.881–2.934	0.122	0.959	0.525–1.750	0.892	0.959	0.543–1.695	0.886
Smoking status										
Never-smokers	169	0.612	0.296–1.264	0.185	1.003	0.488–2.062	0.994	0.904	0.460–1.775	0.769
Ever-smokers	103	10.267	3.374–31.239	<0.001	0.789	0.340–1.829	0.581	0.958	0.417–2.205	0.920

aOR, adjusted odds ratio for age, sex, body mass index, and smoking status; CI, confidence interval.
[a]Categorization defined by the overall median age.

Table 4 Measurement of the interaction between sex, smoking status, and −1195A>G *COX-2* polymorphism in the development and time to diagnosis of colorectal cancer

	Risk estimate					Age at diagnosis		
	Cases *n* (%)	Controls *n* (%)	aOR	95% CI	*P* value	Median	95% CI	*P* value
Females								
Never-smokers								
AA	27 (65.9)	28 (58.3)	1.00	Reference	–	63	(61–65)	0.341
G carriers	14 (34.1)	20 (41.7)	0.48	0.177–1.325	0.158	64	(61–67)	
Ever-smokers								
AA	1 (50.0)	9 (100)	1.00	Reference	–	–	–	–
G carriers	1 (50.0)	0 (0)	–	–	–	–	–	–
Males								
Never-smokers								
AA	20 (74.1)	38 (67.9)	1.00	Reference	–	64	(62–66)	0.462
G carriers	7 (25.9)	18 (32.1)	0.71	0.239–2.096	0.533	69	(54–84)	
Ever-smokers								
AA	22 (46.8)	40 (87.0)	1.00	Reference	–	69	(65–73)	<0.001
G carriers	25 (53.2)	6 (13.0)	9.02	2.940–27.64	<0.001	62	(58–66)	
Risk model								
Not[a]	92 (78.6)	153 (96.2)	1.00	Reference	–	64	(62–66)	0.011
Male ever-smokers G allele carriers	25 (21.4)	6 (3.8)	7.75	2.918–20.58	<0.001	62	(58–66)	

aOR, adjusted odds ratio for age; CI, confidence interval.
[a]Not 'Male ever-smokers G allele carriers'.

Gene–environment interaction

Upon a stratified analysis, we observed a measurable interaction between the −1195G allele and sex (OR: 2.58; 95% CI: 1.290–5.154, $P = 0.007$ in males) or smoking habits although we were only able to gather information about the smoking status in 62% of controls (OR: 10.27; 95% CI: 3.374–31.24, $P < 0.001$ in ever-smokers) (Table 3). More interestingly, the susceptibility for CRC seemed to be modulated by the presence of the G allele particularly in male ever-smokers (OR: 9.01; 95% CI: 2.940–27.64, $P < 0.001$). When evaluating these interactions on the time to diagnosis of CRC, we observed that the waiting time was remarkably lower, once again, in men G allele carriers who ever smoked (62 vs. 69 years, $P < 0.001$) (Table 4). Afterwards, a risk model for CRC was defined considering these individuals and then tested by comparing them with all other participants, and as observed in the bottom of Table 4, a nearly eight-fold higher predisposition for CRC was detected albeit only 31 individuals were included in this model. No association was observed for the remaining two SNPs in the categorized analysis. In addition, no difference in genotype distribution was observed, considering the clinicopathological variables (data not shown).

Discussion

Although the regular use of nonsteroidal anti-inflammatory drugs in cancer prevention is currently jeopardized by the adverse gastrointestinal (GI) complication [16], the anti-inflammatory and antitumor protective effects of their use in CRC prevention, through inhibition of COX-2, are undeniable and consistently observed in several epidemiological and clinical trials [17–19].

In this case–control study, we assessed the role of −1195A > G, −765G > C, and 8473T > C polymorphisms, expected to modulate COX-2 expression, in CRC development not disregarding a possible gene–environment interaction.

As recently reviewed, the −765G > C polymorphism was earlier associated with a higher CRC predisposition in Asiatic populations [8]. We failed to show an association between this polymorphism and CRC risk in our population, which is in agreement with a previous study developed in Caucasians [20]. In contrast, Hoff *et al.* [21] in a larger study observed an increased risk for CRC development in individuals carrying the GG genotype in a Dutch population. The dual and antagonic influence that this polymorphism seems to play in tumor development could have a biological reasoning, as the presence of allele C in *COX-2* promoter region in one instance eliminates the recognition binding site for the Sp1 positive transcription factor leading to a 30% reduction in promoter's activity *in vitro* [9]. In contrast, it also creates an E2F homology binding region that could lead to a higher transcription activity [10]. In fact, the −765CC genotype was associated with a 10-fold enhanced production of PG, compared with the homozygous −765GG [22], thus supporting the observed increased susceptibility to several GI malignancies.

Given its location in the 3′UTR of *COX-2* gene, the 8473T > C polymorphism is a potential candidate to modulate the genetic predisposition for CRC. The thymine (T) to cytosine (C) exchange in an AU-rich elements region, known to control mRNA stability and degradation of several other early intermediate genes encoding inflammatory mediators whose mRNA is very unstable [23], could enhance the mRNA transcripts' stability and ultimately lead to an increased prostaglandins production. In the GI tract, the 8473C allele has been associated with a 1.25-fold increased susceptibility for colorectal adenoma and paradoxically with a protective effect for gastric cancer, compared with 8473GG genotype [8]. Overall, the involvement of this SNP in CRC, however, could not be shown in this study, which is in agreement with the already published studies [20,24,25]. These findings seem to suggest that the 8473T > C polymorphism is more important in early stages of colorectal tumor formation but not so relevant in the progression from adenomatous polyps to malignant tumors.

When focusing on −1195A > G *COX-2* polymorphism, we observed an over-representation of AG and GG genotypes in the group of cases that translated into a 1.7-fold increased predisposition to CRC onset in G allele carriers upon a multivariate analysis. Our results were rather unexpected, although a recently published study involving familial adenomatous polyposis patients also reported an increased risk association between −1195GG genotype and CRC onset [26]. Furthermore, when we assessed possible gene–environment interactions, we observed a positive association between −1195AG/GG genotypes and CRC in males and in ever-active smokers and more interestingly a nine-fold increased predisposition in male ever-smokers. The lack of association in females might be attributable to the small sample size of this group, as only 11% ($n = 11$) of women were current or former smokers. These associations have raised some controversy. In a previous functional study developed by Zhang *et al.* [10], a higher transcriptional activity and increased COX-2 mRNA expression that translated in a significantly higher risk for esophageal cancer in −1195AA genotype carriers was reported [10]. In addition, the AA genotype was further associated with a higher genetic predisposition for the development of gastric and colorectal adenocarcinomas in Asiatic populations [27–29]. These contradictory associations seem to suggest the involvement of other factors that can modulate this polymorphism behavior. In fact, tobacco smoke has been implicated in the colorectal carcinogenesis [30,31] and COX-2 overexpression is suggested as one of the smoke-induced pathways involved in tumor

development [32,33]. Tobacco contains more than 60 identified carcinogens and even though some, such as, nicotine and benzo[a]pyrene, were shown to trigger COX-2 expression through β-adrenoceptors and ERK1/2 pathways, respectively, the pathogenesis of smoking-related CRC is still understudied [33,34]. Hence, it is possible that other smoke-induced pathways may interact with −1195G allele containing promoter region leading to a differential COX-2 expression that only future *in vitro* studies will elucidate. Considering that COX-2-derived PGE-2 is implicated in key steps of tumor development including inhibition of apoptosis, stimulation of angiogenesis, tumor proliferation, and immunosupression [35], it is plausible that functional polymorphisms in *COX-2* gene might influence the time to onset for CRC by modulating COX-2 expression and thus the exposure to PGE-2-induced proneoplastic effects. In fact, we observed that the diagnosis of CRC was anticipated by 7 years in male cases carriers of −1195G allele who ever consumed tobacco, once again reinforcing the role of this *COX-2* SNP in colorectal neoplasia.

These findings represent a preliminary study and as such there are some drawbacks that need to be considered in their interpretation. A major limitation is the missing information on BMI and smoking status in the control group, thus explaining the wider 95% CI observed in the gene–environment interaction analysis. We also cannot exclude selection bias as only 43% of eligible cases were interviewed and informed of this study, mostly explained by the work overload of the clinicians. The sample size of our population, especially the case group, could be insufficient to detect low-magnitude associations and to allow conclusive findings in the stratified analysis. Although we imposed the same age-restriction criteria, we noticed that CRC patients were significantly older than controls. Therefore, we included age as a covariate in the multivariate analysis.

A clearer understanding on CRC etiology through the identification of risk factors might allow a better definition of risk models that are more likely to benefit from preventive strategies. Chan *et al.* [6] reported that the chemopreventive effects of regular use of aspirin in CRC development were exclusively effective in COX-2 overexpressing tumors. In this study, we observed that male ever-smokers carrying at least one −1195G allele had not only a higher genetic predisposition but also were expected to develop CRC at an earlier age, suggesting that this SNP may help predict individuals expected to be exposed to higher levels of COX-2 and thus susceptible to be defined as a risk model for CRC development as observed in this study.

Further research is needed to determine whether this specific subgroup of 'higher-risk' individuals would benefit from the use of COX-2 inhibitors by shifting the balance between costs and benefits that is currently overshadowed by the adverse GI and cardiovascular side-effects.

Acknowledgement

Conflict of interest: none declared.

References

1 Ferlay J, Bray F, Pisani P, Parkin DM. *GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide. IARC CancerBase No. 5, version 2.0.* Lyon: IARC Press; 2004.
2 Giovannucci E. Modifiable risk factors for colon cancer. *Gastroenterol Clin North Am* 2002; **31**:925–943.
3 Weitz J, Koch M, Debus J, Hohler T, Galle PR, Buchler MW. Colorectal cancer. *Lancet* 2005; **365**:153–165.
4 Vane JR, Botting RM. Mechanism of action of nonsteroidal anti-inflammatory drugs. *Am J Med.* 1998; **104**:2S–8S.
5 Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology.* 1994; **107**:1183–1188.
6 Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *N Engl J Med* 2007; **356**:2131–2142.
7 Markowitz SD. Aspirin and colon cancer–targeting prevention? *N Engl J Med* 2007; **356**:2195–2198.
8 Pereira C, Medeiros RM, Dinis-Ribeiro MJ. Cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis: are conclusive results available? *Eur J Gastroenterol Hepatol* 2009; **21**:76–91.
9 Papafili A, Hill MR, Brull DJ, McAnulty RJ, Marshall RP, Humphries SE, *et al.* Common promoter variant in cyclooxygenase-2 represses gene expression: evidence of role in acute-phase inflammatory response. *Arterioscler Thromb Vasc Biol* 2002; **22**:1631–1636.
10 Zhang X, Miao X, Tan W, Ning B, Liu Z, Hong Y, *et al.* Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer. *Gastroenterology* 2005; **129**:565–576.
11 Dixon DA, Kaplan CD, McIntyre TM, Zimmerman GA, Prescott SM. Post-transcriptional control of cyclooxygenase-2 gene expression. The role of the 3′-untranslated region. *J Biol Chem* 2000; **275**:11750–11757.
12 Langsenlehner U, Yazdani-Biuki B, Eder T, Renner W, Wascher TC, Paulweber B, *et al.* The cyclooxygenase-2 (PTGS2) 8473T>C polymorphism is associated with breast cancer risk. *Clin Cancer Res* 2006; **12**:1392–1394.
13 Campa D, Zienolddiny S, Maggini V, Skaug V, Haugen A, Canzian F. Association of a common polymorphism in the cyclooxygenase 2 gene with risk of non-small cell lung cancer. *Carcinogenesis* 2004; **25**:229–235.
14 Pereira C, Sousa H, Ferreira P, Fragoso M, Moreira-Dias L, Lopes C, *et al.* −765G>C COX-2 polymorphism may be a susceptibility marker for gastric adenocarcinoma in patients with atrophy or intestinal metaplasia. *World J Gastroenterol* 2006; **12**:5473–5478.
15 WHO. *Obesity: preventing and managing the global epidemic. Report of a WHO Consultation. WHO technical report series 894.* Geneva: World Health Organization; 2000.
16 Rodriguez LAG TL. Risk of upper gastrointestinal complications among users of traditional NSAIDs and COXIBs in the general population. *Gastroenterology* 2007; **132**:498–506.
17 Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, *et al.* A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med* 2003; **348**:883–890.
18 Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresalier R, *et al.* A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med* 2003; **348**:891–899.
19 Flossmann E, Rothwell PM. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. *Lancet* 2007; **369**:1603–1613.
20 Cox DG, Pontes C, Guino E, Navarro M, Navarro M, Osorio A, *et al.* Polymorphisms in prostaglandin synthase 2/cyclooxygenase 2 (PTGS2/COX2) and risk of colorectal cancer. *Br J Cancer* 2004; **91**:339–343.
21 Hoff JM, te Morsche RHM, Roelofs HMJ, von der Logt EMJ, Nagengast FM. Peters WHM. COX-2 polymorphisms −765G→C and −1195A→G and colorectal cancer risk. *World J Gastroenterol* 2009; **15**:4561–4565.
22 Szczeklik W, Sanak M, Szczeklik A. Functional effects and gender association of COX-2 gene polymorphism G-765C in bronchial asthma. *J Allergy Clin Immunol* 2004; **114**:248–253.

23 Caput D, Beutler B, Hartog K, Thayer R, Brown-Shimer S, Cerami A. Identification of a common nucleotide sequence in the 3′-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc Natl Acad Sci U S A* 1986; **83**:1670–1674.

24 Siezen CL, Bueno-de-Mesquita HB, Peeters PH, Kram NR, van DM, van Kranen HJ. Polymorphisms in the genes involved in the arachidonic acid-pathway, fish consumption and the risk of colorectal cancer. *Int J Cancer* 2006; **119**:297–303.

25 Kury S, Buecher B, Robiou-du-Pont S, Scoul C, Colman H, Le Neel T, *et al*. Low-penetrance alleles predisposing to sporadic colorectal cancers: a French case-controlled genetic association study. *BMC Cancer* 2008; **8**:326.

26 Peters WH, te Morsche RH, Roelofs HM, Mathus-Vliegen EM, Berhout M, Nagengest FM. COX-2 polymorphisms in patients with familial adenomatous polyposis. *Oncol Res* 2009; **17**:347–351.

27 Tan W, Wu J, Zhang X, Liu J, Sun T, Zhang B, *et al*. Associations of functional polymorphisms in cyclooxygenase-2 and platelet 12-lipoxygenase with risk of occurrence and advanced disease status of colorectal cancer. *Carcinogenesis* 2007; **28**:1197–1201.

28 Liu F, Pan K, Zhang X, Zhang Y, Zhang L, Ma J, *et al*. Genetic variants in cyclooxygenase-2: Expression and risk of gastric cancer and its precursors in a Chinese population. *Gastroenterology* 2006; **130**:1975–1984.

29 Zhang XM, Miao XP, Tan W, Sun T, Guo YL, Zhao D, *et al*. [Genetic polymorphisms in the promoter region of cyclooxygenase-2 and their association with risk of gastric cancer]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 2006; **28**:119–123.

30 Botteri E, Iodice S, Bagnardi V, Raimondi S, Lowenfels AB, Maisonneuve P. Smoking and colorectal cancer: a meta-analysis. *JAMA* 2008; **300**:2765–2778.

31 Botteri E, Iodice S, Raimondi S, Maisonneuve P, Lowenfels AB. Cigarette smoking and adenomatous polyps: a meta-analysis. *Gastroenterology* 2008; **134**:388–395.

32 Martey CA, Pollock SJ, Turner CK, O'Reilly KM, Baglole CJ, Phipps RP, Sime PJ Cigarette smoke induces cyclooxygenase-2 and microsomal prostaglandin E2 synthase in human lung fibroblasts: implications for lung inflammation and cancer. *Am J Physiol Lung Cell Mol Physiol* 2004; **287**:L981–L991.

33 Wong HP, Yu L, Lam EK, Tai EK, Wu WK, Cho CH Nicotine promotes colon tumor growth and angiogenesis through beta-adrenergic activation. *Toxicol Sci* 2007; **97**:279–287.

34 Yan Z, Subbaramaiah K, Camilli T, Zhang F, Tanabe T, McCaffery TA, *et al*. Benzo[a]pyrene induces the transcription of cyclooxygenase-2 in vascular smooth muscle cells. Evidence for the involvement of extracellular signal-regulated kinase and NF-kappaB. *J Biol Chem* 2000; **275**:4949–4955.

35 Wang D, Mann JR, DuBois RN. The role of prostaglandins and other eicosanoids in the gastrointestinal tract. *Gastroenterology* 2005; **128**:1445–1461.

CHAPTER II: PURPOSE & AIMS

Considering the findings from the studies enclosed in the previous Chapter I, the purpose with this thesis was to asses the influence of genetic polymorphisms in key genes in PGE_2 pathway (COX-2/HPGD/ABCC4/SLCO2A1) on the susceptibility for the development and recurrence of colorectal tumors that may help identify individuals at higher risk for colorectal lesions that may benefit from targetting screening or be selected for chemopreventive strategies.

Therefore, our aims were:

- To functionally characterize the role of -1195A>G (rs699466) polymorphism in *COX-2* promoter region on gene´s transcriptional activity in CRC cell lines;

- To assess the influence of genetic variants in *COX-2/HPGD/ABCC4/SLCO2A1* genes and possible gene-environment and gene-gene interactions in colorectal cancer development.

- To assess the involvement of polymorphisms in *COX-2/HPGD/ABCC4/SLCO2A1* genes on the risk for development of early stages colorectal lesions and metachronous adenomas and on the time for recurrence.

- To evaluate the influence of polymorphisms proven to influence genetic susceptibility on *COX-2/HPGD/ABCC4/SLCO2A1* mRNA expression.

CHAPTER III: THE -1195G ALLELE INCREASES THE TRANSCRIPTIONALACTIVITY OF *CYCLOOXYGENASE-2* GENE (*COX-2*) IN COLON CANCER CELL LINES

The −1195G Allele Increases the Transcriptional Activity of Cyclooxygenase-2 Gene (COX-2) in Colon Cancer Cell Lines

Carina Pereira,[1,2]* Hugo Sousa,[1] Joana Silva,[1,3] Carla Brandão,[1] Claudio Elgueta-Karstegl,[4] Paul J. Farrell,[4] Rui Medeiros,[1,5] and Mário Dinis-Ribeiro[6,7]

[1]*Molecular Oncology Group, Investigation Centre, Portuguese Institute of Oncology, Porto, Portugal*
[2]*Abel Salazar Institute of Biomedical Sciences, University of Porto, Porto, Portugal*
[3]*Research Department, Portuguese League Against Cancer, Porto, Portugal*
[4]*Section of Virology, Imperial College Faculty of Medicine, London, UK*
[5]*Faculty of Health Sciences of Fernando Pessoa, University of Porto, Porto, Portugal*
[6]*Gastroenterology Department, Portuguese Institute of Oncology, Porto, Portugal*
[7]*Faculty of Medicine, CINTESIS/Department of Biostatistics and Medical Informatics, University of Porto, Porto, Portugal*

Up-regulation of cyclooxygenase-2 (*COX-2*) is an early and key event in human colorectal carcinogenesis (CRC). Nevertheless, the molecular mechanisms leading to this over-expression are largely unknown. We previously reported an association between the −1195G allele and higher predisposition for CRC in a Caucasian population. The biological explanation for the involvement of this polymorphism in CRC remains elusive. We aimed to functionally characterize the influence of the −1195A>G promoter region polymorphism on *COX-2* transcription activity in colon cancer cell lines. Luciferase reporter assays were performed to assess whether the −1195A/G alleles influenced *COX-2* transcription. The *COX-2* promoter's region containing either the −1195A or −1195G alleles was cloned into *pGL3*-basic reporter vector. The reporter vectors were transiently co-transfected with the *pGL4.73* control plasmid to HCT-116 and HCA-7 colon cancer cell lines. The levels of reporter gene expression driven by the −1195G allele-containing *COX-2* promoter were significantly higher in both colon cancer cell lines. A 2.2-fold increase in promoter activity was observed in the HCT-116 cell line ($P < 0.001$), and this over-expression was even more noticeable in the HCA-7 COX-2 expressing cell line with a threefold higher transcriptional activity ($P = 0.001$). The −1195G allele appeared to enhance *COX-2* transcription, providing a molecular basis underlying the increased susceptibility for CRC and potentially a new mechanism for COX-2 overexpression.

Key words: colorectal cancer; genetic susceptibility; cyclooxygenase-2 polymorphism; luciferase assays

INTRODUCTION

Prostaglandin-endoperoxide synthase-2 (PTGS2), most commonly known as cyclooxygenase-2 (COX-2), is considered to be a biomarker of colorectal carcinogenesis, being normally undetectable under physiological conditions and increasingly over-expressed with progression from colorectal adenomas (50%) to CRC (85%) [1]. COX-2-derived prostaglandin E2 (PGE_2) up-regulation is implicated in key steps of carcinogenesis by stimulating cell proliferation, angiogenesis, invasiveness and cell migration, inhibiting apoptosis and modulating immunosuppression [2].

COX-2 gene expression can be controlled by post-transcriptional mechanisms regulating mRNA stability and protein translation, especially at adenylate- and uridylate (AU)-rich elements (ARE) by ARE-binding proteins (HuR, TTP, etc.) or microRNAs (miR-16 and miR-101) [3]. Nevertheless, and although not fully elucidated, the regulation at transcriptional level is also known to be a major mechanism underlying COX-2 up-regulation, through the binding of transcription factors (TF) such as NF-κB, C/EBP, CREB, AP-2, and Sp-1 to several *cis*-acting regulatory elements at *COX-2* promoter [4,5].

In fact, the *COX-2* gene has been shown to be genetically polymorphic, particularly in the 5´ untranslated region, which may lead to differential COX-2 expression [6]. Over the last decade a body of

Abbreviations COX-2, cyclooxygenase; CRC, colorectal cancer; TF, transcription factors; PCR, polymerase chain reaction

Grant sponsor: Portuguese Society of Gastroenterology; Grant sponsor: Portuguese Institute of Oncology of Porto; Grant sponsor: FCT—Fundação para a Ciência e Tecnologia; Grant sponsor: European Social Funds (ESF) under Human Potential Operation Programme (POPH) from National Strategic Reference Framework (NSRF); Grant sponsor: National Strategic Reference Framework (NSRF); Grant number: SFRH/BD/64805/2009

*Correspondence to: Molecular Oncology Group—IPOP Research Centre, Portuguese Institute of Oncology—Porto, Rua Dr. Bernardino de Almeida, 4200-072 Porto, Portugal.

Received 18 February 2013; Revised 22 April 2013; Accepted 3 May 2013

DOI 10.1002/mc.22049

Published online in Wiley Online Library (wileyonlinelibrary.com).

evidence has emerged implicating the involvement of polymorphisms in the *COX-2* promoter in cancer development, although some heterogeneity is noticed among published studies [6,7]. Our group has previously reported not only an increased susceptibility but also a 7-yrs anticipation of CRC development for G allele carriers of the −1195A>G *COX-2* promoter region polymorphism [8]. These results are in agreement with the increased-risk trend observed for colonic adenoma onset associated with the −1195GG genotype in Japanese and Dutch populations [9,10]. However, earlier studies in an Asiatic population implicated the −1195AA genotype as a risk marker for digestive cancers development (gastric, oesophageal, and colorectal) [11–13]. The controversial evidence arising from both epidemiological and experimental studies addressing the functional repercussion of this polymorphism on COX-2 expression in different models suggests that different pathways might be activated depending on cell type, tissue or pathological conditions [12,14]. So far, the biological explanation of the −1195A>G polymorphism in colon carcinogenesis remains elusive. Therefore, with this in vitro study we aimed to functionally characterize the influence of the −1195A>G promoter region polymorphism on *COX-2* transcription activity in colon cancer cell lines.

MATERIALS AND METHODS

Cell Lines Culture

The human colon cancer cell lines HCT116 and HCA-7 were purchased from the European Collection Cell Cultures (ECACC) as they derive from colon carcinoma tissues and had previous COX-2 expression data available. They were cultured in a humidified, 5% CO_2 incubator at 37°C in McCoy's 5A with GlutaMAX and Dulbecco's modified Eagle medium (DMEM) with GlutaMAX, respectively, supplemented with 10% heat-inactivated fetal bovine serum, Penicillin (50 U/ml), and Streptomycin (0.05 mg/ml). All reagents were acquired from Gibco (Life Technologies Corporation, Carlsbad, CA).

Reporter Plasmids Constructs

A 1706 bp DNA fragment from the *COX-2* promoter region containing the −1195A allele was amplified with following primers: F: 5′ AATATGACTAGAGGAGGAGAAAGG 3′ and R: 5′ GGAAGCTTAGGCTTTGCTGTCTGAG 3′, which included a *Hin*dIII restriction site (underlined sequence) and sub-cloned in the pCR2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen, Life Technologies Corporation).

The −1195G allele was obtained by site-directed mutagenesis using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies, Inc, Santa Clara, CA) and confirmed by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP; Figure 1). To ensure their authenticity and exclude mis-incorporations during the mutagenesis both plasmids were sequenced using the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Life Technologies Corporation).

The pCR2.1-TOPO vector plus inserts were then double digested with *Kpn*I and *Hin*dIII restriction enzymes (New England Biolabs, Ipswich, MA) and the inserts cloned into an appropriate double digested *pGL3*-basic vector (Promega, Madison, WI), encoding the firefly luciferase protein as reporter. The ligation was obtained after a 14°C overnight incubation with T4 ligase using a 3:1 inserts to vector ratio (Promega) and confirmed by PCR with intragenic primers used to characterize the −1195A>G polymorphism [12].

Transient Transfection and Measurement of Luciferase Activity

The HCT116 and HCA-7 cell lines were seeded in 96-well plates (6×10^4 and 1×10^5 cells/well, respectively) and grown to 90−95% confluency. After a 24 h incubation the cells were transfected using the Lipofectamine 2000 transfection reagent (Invitrogen, Life Technologies Corporation) following manufacturer's instructions. Briefly, 200 ng of reporter plasmids was co-transfected with 1.33 ng of *pGL4.73* vector (Promega) to HCT116 and HCA-7 cells to normalize transfection efficiency. The promoter activity was quantified using the Dual-Glo Luciferase Assay System (Promega) 24 h after transfection. Cell viability was assessed by staining the trypsinized cells with 0.4% trypan blue (Gibco, Life Technologies Corporation) and counting the viable cells in a Neubauer chamber. Over 90% cell viability was observed when measuring Luciferase activity.

Statistical Analysis

Fold increase was measured by defining the activity of −1195A allele-containing plasmid as 1. Data shows the means fold increase + SD from two independent transfection experiments performed in triplicate. The unpaired *t*-test was used to assess the difference between promoters' activity, with a significance value of $P < 0.05$.

RESULTS

We evaluated the effect of the different alleles of −1195A>G polymorphism in *COX-2* transcriptional activity by transiently transfecting luciferase reporter vector containing either allele to HCT116 and HCA-7 colon cancer cell lines.

The promoter activity of *COX-2* showed an allele-specific behaviour. The levels of reporter gene expression driven by the −1195G allele-containing *COX-2* promoter were significantly higher in both colon cancer cell lines. A 2.2-fold increase in promoter activity was observed in comparison to the −1195A allele-containing counterparts in the HCT-116 cell

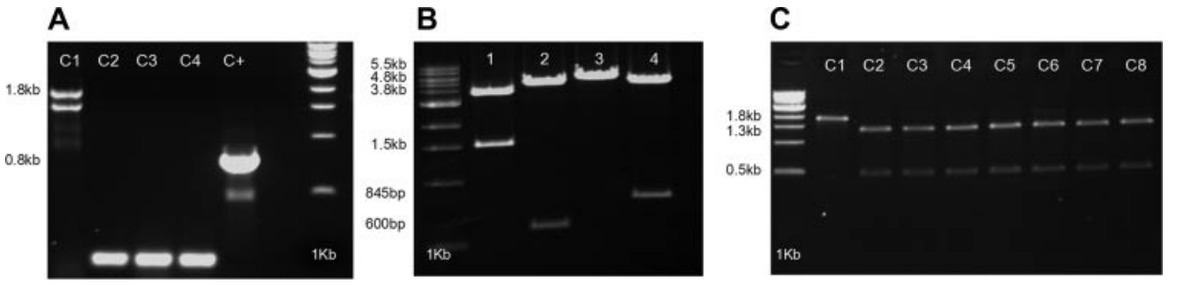


Figure 1. Confirmation of subcloning of −1195A and −1195G allele-containing *COX-2* promoter in pCR2.1-TOPO vector. (A) Colony-PCR with T7 promoter and M13 reverse primers to screen the colonies for the desired plasmid. C1: positive colony (1.8 kb fragment); C2−C4, false positive; C+, positive control (0.8 kb band); (B) Map restriction of pCR2.1-TOPO plus insert to confirm its authenticity using: (1) *Kpn*I and *Hin*dIII restriction enzymes: 3.8 kb + 1.5 kb + 160 bp + 10 pb; (2) *Kpn*I and *Sac*I: 4.8 kb + 600 bp + 200 bp + 6 bp; (3) *Kpn*I: 5.5 kb + 160 bp; and (4) *SacI*: 4.8 kb + 845 bp; (C) Identification of colonies transformed with the mutated allele (−1195G allele) after site-directed mutagenesis (Stratagene, Agilent Technologies, Inc) through PCR-RFLP. C1: −1195A allele (undigested fragment, 1.8 kb); C2−C8: −1195G allele (the G allele is recognized by *Pvu*II restriction enzyme (New England Biolabs, Ipswich, MA) creating a 1.3 kb + 0.5 kb fragments) [12].

line ($P < 0.001$) that was even more noticeable in the HCA-7 COX-2 expressing cell line (threefold higher transcriptional activity for those with −1195G allele, $P = 0.001$).

DISCUSSION

Up-regulation of cyclooxygenase-2 (COX-2) is an early and key oncogenic event in human colorectal carcinogenesis [1]. Nevertheless, the molecular mechanisms leading to this over-expression are largely unknown.

Earlier epidemiological studies have implicated the −1195A>G *COX-2* polymorphism in colorectal cancer development [8,13]. Although it is one of the most studied polymorphisms in the *COX-2* gene, its biological role in CRC remains elusive.

With this experimental study, we demonstrated that *COX-2* promoter containing the −1195G allele leads to a higher transcription activity in contrast with the −1195A allele in colorectal cancer cell lines. The difference in promoter activity between the HCT116 and HCA-7 cell lines may reflect the molecular heterogeneity observed among colon tumors. While the HCA-7 cell line constitutively expresses high levels of COX-2, the HCT-116 lacks this protein expression [15].

The *COX-2* gene has a promoter region rich in *cis*-acting regulatory elements and a complex network of pathways and nuclear proteins involved in its transcription regulation [4,5]. Hence, it is biologically plausible that the −1195A−G substitution might modify the recognition binding sites or influence the binding affinity of specific TF, as observed with a previous genetic polymorphism in *COX-2* promoter [16]. Zhang et al. [12], initially showed that the presence of −1195A allele creates a recognition binding site for the c-myb TF that led to a four- to sixfold increase in promoter's activity in HeLa cells, which was further reproduced in a gastric cancer cell line (AGS) particularly when stimulated with homogenated tissue infected with *Helicobacter pylori* [11]. This behaviour was further corroborated by the enhanced expression of COX-2 mRNA in oesophageal tissues [12]. On the other hand, Sakaki et al. [15]. using human hepatoma cell lines (HepG2 and Huh-7) reported a higher transcriptional activity in cells transfected with −1195G allele-containing promoter than the ones with −1195A allele. These results suggest that this polymorphism in *COX-2* promoter region might have a histological/cell-dependent behavior, possibly modulated by the activation of pathways and nuclear proteins specific to each disease model.

This report is the first study to evaluate the influence of −1195A>G polymorphism on *COX-2* promoter activity in colon carcinogenesis. A bioinformatics analysis using the DS Gene software (version 1.1, Accelrys Inc., Cambridge, UK), predicted that, besides eliminating a c-myb binding site, the −1195A>G substitution also creates an E-box motif, frequently found in genes that are highly expressed in colon [17]. The E-box motif is recognized by several TFs, namely, the upstream stimulator factor 1 (USF1), previously shown to positively regulate the transcription of several genes involved in colon cancer development [18–20]. This might provide additional clues for future functional studies to unravel the molecular mechanism leading to the increased promoter activity in the presence of the −1195G allele in colon cancer lines and higher susceptibility for CRC development.

In conclusion, with this experimental study we demonstrated that the −1195G allele increases *COX-2* transcription, providing a biological reasoning underlying the higher susceptibility previously reported for CRC and potentially a new mechanism for COX-2 overexpression that could be investigated further in future studies.

ACKNOWLEDGMENTS

This study was supported by research grants from the Portuguese Society of Gastroenterology and the Portuguese Institute of Oncology of Porto. Furthermore,

C.P. is a recipient of a PhD grant (SFRH/BD/64805/2009) from FCT—Fundação para a Ciência e Tecnologia, co-financed by European Social Funds (ESF) under Human Potential Operation Programme (POPH) from National Strategic Reference Framework (NSRF) and had an allowance for supplemental training by the Portuguese League Against Cancer—regional Centre of the North.

We also acknowledge Dr. Paula Paulo for the help provided during the sequencing of plasmids.

REFERENCES

1. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107:1183–1188.
2. Wang D, Mann JR, Dubois RN. The role of prostaglandins and other eicosanoids in the gastrointestinal tract. Gastroenterology 2005;128:1445–1461.
3. Dixon DA, Blanco FF, Bruno A, Patrignani P. Chapter 2: Mechanistic aspects of COX-2 expression in colorectal neoplasia. Recent Results Cancer Res 2013;191:37–37.
4. Appleby SB, Ristimaki A, Neilson K, Narko K, Hla T. Structure of the human cyclo-oxygenase-2 gene. Biochem J 1994;302:723–727.
5. Kosaka T, Miyata A, Ihara H, Hara S, Sugimoto T, Takeda O. Characterization of the human gene (PTGS2) encoding prostaglandin-endoperoxide synthase 2. Eur J Biochem 1994;221:889–897.
6. Pereira C, Medeiros R, Pereira C. Cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis: Are conclusive results available? Eur J Gastroenterol Hepatol 2009;21:76–91.
7. Dong J, Dai J, Zhang M, Hu Z, Shen HJ. Potentially functional COX-2 −1195G>A polymorphism increases the risk of digestive system cancers: A meta-analysis. Gastroenterol Hepatol 2010;25:1042–1050.
8. Pereira C, Pimentel-Nunes P, Brandão C, Moreira-Dias L, Medeiros R, Dinis-Ribeiro M. COX-2 polymorphisms and colorectal cancer risk: A strategy for chemoprevention. Eur J Gastroenterol Hepatol 2010;22:607–613.
9. Ueda N, Maehara Y, Tajima O, Tabata S, Wakabayashi K, Kono S. Genetic polymorphisms of cyclooxygenase-2 and colorectal adenoma risk: The Self Defense Forces Health Study. Cancer Sci 2008;99:576–581.
10. Peters WH, te Morsche RH, Roelofs HM, Mathus-Vliegen EM, Berhout M, Nagengest FM. COX-2 polymorphisms in patients with familial adenomatous polyposis. Oncol Res 2009;17:347–351.
11. Liu F, Pan K, Zhang X. et al. Genetic variants in cyclooxygenase-2: Expression and risk of gastric cancer and its precursors in a Chinese population. Gastroenterology 2006;130:1975–1984.
12. Zhang X, Miao X, Tan W. et al. Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer. Gastroenterology 2005;129:565–576.
13. Tan W, Wu J, Zhang X. et al. Associations of functional polymorphisms in cyclooxygenase-2 and platelet 12-lipoxygenase with risk of occurrence and advanced disease status of colorectal cancer. Carcinogenesis 2007;28:1197–1201.
14. Sakaki M, Makino R, Hiroishi K. et al. Cyclooxygenase-2 gene promoter polymorphisms affect susceptibility to hepatitis C virus infection and disease progression. Hepatol Res 2010;40:1219–1226.
15. Sheng H, Shao J, Kirkland SC. et al. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J Clin Invest 1997;99:2254–2259.
16. Zhao D, Xu D, Zhang X. et al. Interaction of cyclooxygenase-2 variants and smoking in pancreatic cancer: A possible role of nucleophosmin. Gastroenterology 2009;136:1659–1668.
17. Suzuki Y, Tsunoda T, Sese J. et al. Identification and characterization of the potential promoter regions of 1031 kinds of human genes. Genome Res 2001;11:677–684.
18. Bélanger AS, Tojcic J, Harvey M, Guillemette C. Regulation of UGT1A1 and HNF1 transcription factor gene expression by DNA methylation in colon cancer cells. BMC Mol Biol 2010; 11.
19. Ansorge N, Jüttner S, Cramer T, Schmidt WE, Höcker M, Schmitz F. An upstream CRE-E-box element is essential for gastrin-dependent activation of the cyclooxygenase-2 gene in human colon cancer cells. Regul Pept 2007;144:25–33.
20. Jaiswal AS, Narayan S. Upstream stimulating factor-1 (USF1) and USF2 bind to and activate the promoter of the adenomatous polyposis coli (APC) tumor suppressor gene. J Cell Biochem 2001;81:262–277.

CHAPTER IV: GENETIC VARIABILITY IN KEY GENES IN PROSTAGLANDIN E_2 PATHWAY (*COX-2, HPGD, ABCC4* AND *SLCO2A1*) AND THEIR INVOLVEMENT IN COLORECTAL CANCER DEVELOPMENT

OPEN ACCESS Freely available online

PLOS ONE

Genetic Variability in Key Genes in Prostaglandin E_2 Pathway (*COX-2, HPGD, ABCC4* and *SLCO2A1*) and Their Involvement in Colorectal Cancer Development

Carina Pereira[1,2,3]*, Sara Queirós[1], Ana Galaghar[4], Hugo Sousa[1], Pedro Pimentel-Nunes[5,6], Catarina Brandão[5], Luís Moreira-Dias[5], Rui Medeiros[1,2,3,7], Mário Dinis-Ribeiro[5,8]

1 Molecular Oncology Group, Investigation Centre, Portuguese Institute of Oncology, Porto, Portugal, **2** Abel Salazar Institute of Biomedical Sciences, University of Porto, Porto, Portugal, **3** Research Department, Portuguese League Against Cancer, Porto, Portugal, **4** Pathology Department, Portuguese Institute of Oncology, Porto, Portugal, **5** Gastroenterology Department, Portuguese Institute of Oncology, Porto, Portugal, **6** Physiology Department, Faculty of Medicine, University of Porto, Porto, Portugal, **7** CEBIMED, Faculty of Health Sciences of Fernando Pessoa University of Porto, Porto, Portugal, **8** CINTESIS/Department of Biostatistics and Medical Informatics, Faculty of Medicine, University of Porto, Porto, Portugal

Abstract

The pro-carcinogenic effects of prostaglandin E2 (PGE_2) in colonic mucosa are not only regulated by the rates between Cyclooxygenase-2 (COX-2) biosynthesis and 15-Hydroxyprostaglandin Dehydrogenase (15-PGDH)-dependent degradation but also the steady-state levels of PGE_2 in extracellular microenvironment, maintained by key specific prostaglandin transporters, the Multidrug Resistance Protein (MRP4) (efflux carrier) and Prostaglandin Transporter (PGT) (influx carrier). To understand the contribution of genetic variability in genes coding for COX-2/15-PGDH/MRP4/PGT proteins in CRC development, we conducted a hospital-based case-control study involving 246 CRC patients and 480 cancer-free controls. A total of 51 tagSNPs were characterized using the Sequenom platform through multiplexed amplification followed by mass-spectrometric product separation or allelic discrimination using real-time PCR. Seven tagSNPs were implicated in CRC development: the rs689466 in *COX-2* gene, the rs1346271 and rs1426945 in 15-PGDH, the rs6439448 and rs7616492 in PGT and rs1751051 and rs1751031 in MRP4 coding genes. Upon a stratified analysis a measurable gene-environment interaction was noticed between rs689466 and smoking habits, with individuals ever-smokers carriers of rs689466 GG homozygous genotype having a nearly 6-fold increased susceptibility for CRC onset (95%CI: 1.49–22.42, $P = 0.011$). Furthermore, the multifactor dimensionality reduction (MDR) analysis identified an overall four-factor best gene-gene interactive model, including the rs1426945, rs6439448, rs1751051 and rs1751031 polymorphisms. This model had the highest cross-validation consistency (10/10, $P<0.0001$) and an accuracy of 0.6957 and was further associated with a 5-fold increased risk for CRC development (95%CI: 3.89–7.02, $P<0.0001$). In conclusion, specific low penetrance genes in the pro-carcinogenic PGE_2 pathway appear to modulate the genetic susceptibility for CRC development. A clearer understanding on CRC etiology through the identification of biomarkers of colorectal carcinogenesis might allow a better definition of risk models that are more likely to benefit from targeted preventive strategies to reduce CRC burden.

Citation: Pereira C, Queirós S, Galaghar A, Sousa H, Pimentel-Nunes P, et al. (2014) Genetic Variability in Key Genes in Prostaglandin E_2 Pathway (*COX-2, HPGD, ABCC4* and *SLCO2A1*) and Their Involvement in Colorectal Cancer Development. PLoS ONE 9(4): e92000. doi:10.1371/journal.pone.0092000

Editor: Kjetil Tasken, University of Oslo, Norway

Received November 25, 2013; **Accepted** February 15, 2014; **Published** April 2, 2014

Funding: This study was supported by a research grant from the Portuguese Institute of Oncology of Porto. Furthermore, CP is a recipient of a PhD grant (SFRH/BD/64805/2009) from FCT-Fundacão para a Ciência e Tecnologia, co-financed by European Social Funds (ESF) under Human Potential Operation Programme (POPH) from National Strategic Reference Framework (NSRF). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflicts: RM is a PLoS ONE Editorial Board member. This does not alter the authors' adherence to PLOS ONE Editorial policies and criteria.

* E-mail: anacmpereira@gmail.com

Introduction

Colorectal cancer (CRC) is the most widespread malignancy in developed regions, accounting for over 13% of all diagnosed cases (728.550 cases) and 11% of all cancer-related deaths in 2008 (320.279 deaths) [1]. The burden of CRC is increasing as a reflection of population growth and aging, also as, an increased adoption of cancer-associated "westernized" lifestyle [2]. So, the implementation of population-based CRC screening guidelines focusing on the detection and removal of precancerous lesions is highly recommended for a successful decrease in CRC incidence rates [3]. Unfortunately, the compliance rates are far from the desirable and considerably lower than those reported for other recommended preventive strategies [4], which compromises the efficacy of these approaches in CRC prevention. This might provide reasoning not only for targeted screening but also the pursuit for alternative and/or complementary strategies, namely the use of chemoprevention to significantly reduce this cancer burden.

One group of compounds with extensive data supporting their preventive role in cancer onset include the nonsteroidal anti-inflammatory drugs (NSAIDs), shown to reduce the relative risk of developing CRC by 40–50%, mainly by targeting the cyclooxygenase-2 (COX-2) enzyme [5–7].

COX-2 is an immediate-early response gene, previously shown to be up-regulated in 40–50% of colorectal adenomas and 85% of CRC, leading to the extracellular microenvironment accumulation of prostaglandins (PGs) [8]. COX-2-derived PGE_2, the major PG produced in colorectal tumors, plays a key contribution to the hallmarks of cancer, by stimulating cell proliferation, invasiveness and migration, enhancing angiogenesis, evading apoptosis and modulating the antitumor immune response [9]. COX-2 has a physiologic antagonist in 15-hydroxyprostaglandin dehydrogenase (15-PGDH) that catabolizes PGE_2 to an inactive keto compound [10]. 15-PGDH is highly expressed in normal mucosa and one of the most down-regulated genes in colorectal tumors, being a potent *in vivo* suppressor of colon neoplasia by decreasing the catabolism of PGE_2 [11,12]. Furthermore, low 15-PGDH levels are associated with resistance to COX-2 selective inhibitor Celecoxib chemopreventive effects in colorectal tumors development, reinforcing the impact of loss of 15-PGDH expression in colorectal carcinogenesis [13]. Notwithstanding, the biologic effects of the COX-2/PGE_2 pathway are not only regulated by the rates between COX-2 biosynthesis and 15-PGDH-dependent degradation but also the steady-state levels of PGE_2 in extracellular microenvironment, regulated by key specific prostaglandin transporters [14,15]. The multidrug resistance-associated protein 4 (MRP4) is responsible for exporting PGE_2 into the extracellular milieu, where a plethora of pathways will be activated through binding to specific G-protein couple receptors [14]. On the other hand, the active uptake back into the cytoplasm, where PGE_2 will be inactivated by 15-HPGD, is carried-out by prostaglandin transporter (PGT) [15]. In fact, Holla and colleagues [16] reported that PGT and MRP4 mRNA levels are inversely regulated in human CRC, with PGT expression being downregulated and MRP4 overexpressed in CRC tissues and cell lines leading to higher levels of PGE_2 extracellularly thus upregulating the effects of COX-2/PGE_2 pathway.

A decade ago the release of the first human genome draft allowed a deeper knowledge on the architecture and function of the human genome, highlighting the relevance of common genetic variations on disease genesis. In CRC, family history is a well-established etiologic factor, shedding some clues for the involvement of low penetrance genes in its oncogenesis [17].

The *COX-2* gene is genetically polymorphic and was the target of several genetic association studies, implicating the involvement of three polymorphism in *COX-2* gene on colorectal tumors development (rs20417, rs699466 and rs5275, also known as −765G>C, −1195A>G and 8473T>C, respectively), although not always consistently [18]. In a preliminary study, we reported an increased susceptibility for CRC development in G allele carriers of the rs689466A>G polymorphism in *COX-2* promoter's [19].

Hoeft and colleagues [20] firstly identified two tagging single nucleotide polymorphisms (tagSNPs), the rs8752 and rs2612656 in *HPGD* gene, coding for the 15-PGDH protein, as increased susceptibility markers for CRC development. More recently, Thompson and colleagues [21] observed a 40% increased risk associated with the rs2555639 SNP located at 17.74 kb upstream of the 5′UTR of *HPGD* gene that was further validated in the replication set.

With the exception of a two-phase case-control study in a Spanish population [22] no previous study inquired the role of common genetic variants in MRP4 and PGT coding genes (*ATP-Binding Cassette Sub-Family C Member 4* (*ABCC4*) and *solute carrier organic anion transporter family, member 2A1* (*SLCO2A1*), respectively) in CRC genesis. Neither addressed the combined effect of SNPs in these four genes with pivotal roles in modulating the levels of PGE_2 extracellularly. So, in this case-control study we explored the associations of 51 common genetic variations in *COX-2/HPGD/ABCC4/SLCO2A1* PGE_2 pathway genes with CRC onset.

Materials and Methods

Sample Size Estimation

We estimated that the sample size required to detect an Odds Ratio (OR) equal or superior to 1.70 is 200 patients and 400 controls (2:1 ratio) to achieve a statistical power of 80%, with a significance level of 5%, for polymorphisms with a frequency superior to 15%. (Epi Info version 6, Centers for Disease Control, Atlanta, Georgia). Considering that r^2, used to select the tagSNPs, is inversely related to the magnitude by which the sample size must be increased in a study design, for a r^2 of 0.8 we needed to increase our sample size by 25%.

Study Population

This non-matched hospital-based case-control study included 726 participants: 246 histologically confirmed CRC patients and 480 cancer-free controls, from the northern region of Portugal and recruited at the *Instituto Português de Oncologia do Porto* (IPO-Porto).

Written informed consent was obtained from all recruited participants before their inclusion in the study, according to the Declaration of Helsinki. This research project was approved by the Ethics Committee of the IPO-Porto (ref. 0084/08) and *Comissão Nacional de Protecção de Dados* (ref. 6619/2011) that is the Portuguese Data Protection Authority.

Control group. In this group, individuals between 50 and 75 years of age, without any clinical evidence of CRC or other oncologic malignancy were randomly recruited from the blood donor's service at IPO-Porto between July 2005 and February 2008.

CRC patients group. Patients with histologically confirmed CRC newly diagnosed between January 2002 and September 2007 were enrolled in this study. These patients were selected from a colonoscopy database from the Gastroenterology Department, aged 50 to 75 years, without previous history of inflammatory bowel disease or hereditary syndromes and who were scheduled for a follow-up consult at *Serviço de Gastrenterologia* or *Unidade de Digestivos* at IPO Porto between March and May 2008.

Two hundred and forty seven CRC patients were included out of the 387 expected to be recruited. During the recruitment or afterwards by telephone interview patients were asked to recall their lifestyle habits (smoking behavior, BMI, etc) in the previous year of CRC diagnosis. Medical records were reviewed to extract the clinicopathological variables (stage, tumor grade, presence of synchronous and metachronous lesions) and to exclude misclassification bias.

Sample Collection and Biological Processing

Blood samples were collected using standard venipuncture technique with EDTA containing tubes. DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

For patients unable to provide a blood sample, the DNA was extracted from formalin fixed paraffin embedded (FFPE) blocks from the Pathology Department at our institute. Two to four 10 μm thickness section were used in each extraction depending on the size of tissue area (1.5–3 cm^2). Briefly, the CRC tissue specimens from each glass slide were scraped, using a clean razor blade, into a 1,5-ml microcentrifuge tube. The samples were deparaffinised in xylene for 10 minutes, at room temperature,

followed by centrifugation at 14.000 g–16.000 g for 3 minutes. The tissue pellets were then rehydrated with 1 ml of absolute ethanol, followed by centrifugation at 14.000 g–16.000 g for 3 minutes and the supernatant was discarded. This step was repeated twice. Then, the tube was maintained open for 15 minutes to evaporate any remaining ethanol. Further steps of DNA isolation were performed using the GRS Genomic DNA Kit – Tissue, in accordance with the manufacturer's protocol (GRiSP, Porto, Portugal).

DNA was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and stored at $-20^{\circ}C$ until genotype examination. The DNA quality was determined by measuring the optical density (OD) 260/280 ratio.

Validation of DNA Genotyping Extracted from FFPE Samples

To assess whether DNA isolated from FFPE sections is reliable for retrospective genotyping we compared the genotypes of 20 somatic DNAs extracted from FFPE specimens to germline DNAs isolated from fresh peripheral blood from the same patients. The genotypes were highly concordant (100%).

Polymorphisms Selection

Using a tagSNP approach, the genetic variants were retrieved from a set of common SNPs in the Caucasian population of HapMap project (CEU). The Genome Variation Server (version 7.00) was used to recover tagSNPs capturing variations (1) with a minor allele frequency equal or superior to 15%; (2) within the coding region of the genes plus 2 Kb upstream and downstream and (3) with a r^2 superior to 0.8. A total of 140 tagSNPs were captured: 6, 15, 31 and 88 tagSNPs in *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* genes, respectively. We further selected SNPs with high likelihood of genotyping success using the Sequenom platform, (Sequenom, San Diego, CA). Briefly, tagSNPs were prioritized as follows: first, all non-singletons tagSNPs or singletons with expected functional repercussion (FuncPred software) were tested. TagSNPs with low genotyping scores were replaced with representative variants; and finally the non-significant singletons were included in the array design. A total of 55 SNPs were successfully converted to the Sequenom platform.

Furthermore, we also included polymorphisms that were previously associated with colorectal tumors development and had a minor allele frequency equal or superior to 15% that failed to converted to the Sequenom platform: rs20417, rs689466 and rs5275 in *COX-2* and rs2612656 and rs2555639 in *HPGD* genes.

Genotype Characterization

TagSNP genotyping was performed using MassARRAY iPLEX Gold technology (Sequenom, San Diego, CA) based on multiplexed amplification followed by mass-spectrometric product separation. This technique was carried-out by the *Unidade de Genómica/Serviço de Genotipagem do Instituto Gulbenkian de Ciência*.

All polymorphisms not included in the tagSNPs analysis were characterized through allelic discrimination (Real-Time Polymerase Chain Reaction) using validated TaqMan® SNP genotyping assays (C___2517145_20, C___7550203_10, C___15909858_20, C___16038735_10 for the rs689466, rs5275, rs2612656 and rs2555639, respectively) with the exception of the polymorphism −765G>C (rs20417) which was custom designed (Applied Biosystems, Foster City, California USA). Allelic discrimination was performed by measuring end-point fluorescence using ABI PRISM Sequence Detection System (Applied Biosystems, Foster City, California USA).

Quality Control

Genotypes were excluded from the analysis if any of the following criteria was applied: call rate inferior to 0.90; concordance rate inferior to 0.95 and Hardy-Weinberg equilibrium (HWE) with $P<0.05$. Blank templates were included in each 96 and 384-well plates to ensure contamination-free results. Two researchers performed the genotype interpretation independently and five to ten percent of all samples were randomly selected and re-submitted to a new genetic characterization to confirm the genotypes.

Statistical Analysis

For genetic distribution analysis, the Hardy–Weinberg equilibrium was tested by the Pearson's goodness-of-fit test to compare the observed versus the expected genotype distribution among the control population.

Data analysis was performed using the computer software IBM Statistical Package for Social Sciences-SPSS (IBM Corp., Armonk, New York, USA) for Macintosh (version 19.0). Chi-square analysis was used to compare categorical variables, using a 5% level of significance. Non-parametric tests were used to compare mean values. Odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measure of the association between the genetic variants and the risk for the development of CRC. Covariates proven to differ between group populations were included in the logistic regression analysis. Gene-environment interaction analysis were carried-out by stratifying data considering the gender, smoking habits and body mass index (BMI). Additionally, a bootstrap resampling was used to investigate the stability of risk estimates (1000 replications). Furthermore, the false positive report probability (FPRP) was used to confirm the noteworthiness of significant findings, according to the study by Wacholder and colleagues [23]. The FPRP threshold was set at 0.5 under an assigned prior probability ranging from 0.01 to 0.25 to detect an OR of 1.5.

Haplotype analysis was performed at a gene level using the SNPStats software (www. http://bioinfo.iconcologia.net/SNPstats). The haplotype frequencies were estimated using the implementation of the EM algorithm coded into the *haplo.stats* package. The most frequent haplotype was automatically selected as the reference category. For the *HPGD*, *SLCO2A1* and *ABCC4* genes the haplotype blocks were constructed considering the most meaningful polymorphisms.

The open-source multifactor dimensionality reduction (MDR) software (version 3.0.2) (www.epistasis.org) was used to assess potential gene-gene interactions between SNPs with statistical significant impact on CRC genetic susceptibility. The fitness of an MDR model was estimated by determining the testing accuracy and its cross-validation consistency (CVC). Using a 10-fold cross-validation method the data was divided into 10 sets, in which 9 subsets were training sets and one subset was a test set. Hence, the CVC is a measure of the number of times of 10 divisions of the dataset the best model was extracted. The single best model normally has the maximal testing accuracy and CVC. Statistical significance was evaluated using a 1000-fold permutation test to compare observed testing accuracies with those expected under the null hypothesis of null association. Permutation testing corrected for multiple testing by repeating the entire analysis on 1000 datasets that were consistent with the null hypothesis.

Results

Description of Study Population

The characteristics of the study population are summarized in Table 1. Cases were significantly older than controls with a median age of 63 years (50–75) (vs. 58 years in controls (50–69), $P<0.001$). Males were overrepresented in both groups (60.1% vs 65.4% in cases and controls, respectively, $P=0.159$) and nearly 77% of participants were overweight ($P=0.955$). The majority of participants had also never smoked in either category (37.4% in cases and 39.7% in controls, $P=0.636$).

Genotype Frequencies and Risk Estimates

Three SNPs and four samples were excluded from the analysis due to genotyping failure and four SNPs were dropped because their frequencies deviated from HWE ($P<0.05$). A total of 51 SNPs were included in the risk estimate analysis. The mean genotype call and concordance rates were 99.02% and 99.3%, respectively. The description of selected SNPs is displayed in Table S1.

Overall seven genetic polymorphisms across the four genes were implicated in colorectal carcinogenesis, as can be observed in Table 2. The AG and GG genotypes of the rs689466 polymorphism in *COX-2* gene were overrepresented in the group of cases leading to an increased risk for CRC more noticeable for homozygous GG although this was not statistically significant in the multivariate analysis (OR = 2.01; 95%CI:0.93–4.35, $P=0.076$). The rs1346271 and rs1426945 SNPs in *HPGD* gene were associated with a 32% and 44% decreased risk for CRC onset (95%CI:0.47–0.96, $P=0.029$ and 95%CI:0.34–0.93, $P=0.026$, for the GC and AA homozygous carriers of the rs1346271 and rs1426945 polymorphisms, respectively). Out of the fifteen genetic variations analyzed in the *SLCO2A1* gene only the rs6439448 and rs7616492 polymorphisms influenced the

Table 1. Description of participants.

	Cases	Controls	
	(n = 246)	**(n = 480)**	***P* value**
Demographics			
Age (years)			
Mean (SD)	63 (7.2)	58 (4.9)	<0.001
Median (min–max)	63 (50–75)	58 (50–69)	
Sex, n (%)			
Male	146 (60.1)	314 (65.4)	0.159
Female	97 (39.9)	166 (34.6)	
Lifestyle behavior			
BMI (Kg/m^2)			
Mean (SD)	28 (4.2)	28 (3.6)	0.510
Median (min–max)	28 (20–43)	27 (20–41)	
BMI category, n (%)[#]			
<25	34 (23.4)	48 (23.2)	0.955
≥25	111 (76.6)	159 (76.8)	
Smoking status, n (%)			
Never-smokers	92 (62.6)	219 (60.3)	0.636
Ever-smokers*	55 (37.4)	144 (39.7)	
Tumor characteristics			
Tumor location, n (%)			
Rectum	127 (52.3)	–	
Colon	116 (47.7)	–	
Stage, n (%)			
I–II	121 (52.6)	–	
III–IV	109 (47.4)	–	
Grade, n (%)			
Low grade	135 (95.7)	–	
High grade	6 (2.4)	–	
Synchronous tumors, n (%)			
Yes	14 (5.5)	–	
No	224 (88.2)	–	

BMI, body mass index;
[#]Categorization based on the cutoff defined by the world Health Organization for overweight people;
*Never- and former-smokers pooled together; For synchronous tumors the most advanced lesions was the one considered in the tumors' characterization.
doi:10.1371/journal.pone.0092000.t001

susceptibility for CRC. Individuals carriers of the rs6439448 heterozygous AG genotype presented an OR of 0.68 (95%CI:0.47–0.99, $P = 0.047$). On the other hand, a two-fold increased predisposition was noticed for individuals carrying both copies of the A allele of rs7616492 polymorphism (95%CI:1.27–3.32, $P = 0.003$). Focusing on *ABCC4* gene, a 1.76 enhanced susceptibility was observed with the AA genotype of rs1751051 SNP and a protection was evident for AG genotype carriers of rs1751031 polymorphism (OR = 0.68; 95%CI:0.47–0.97, $P = 0.032$). The bootstrap analysis supported our results (Table 2). The genotypes distribution of all included SNPs is reported in Table S2.

The FPRP analysis revealed that the unadjusted significant associations observed in Table 2, retained their significance (FPRP≤0.5) when a prior probability equal or superior to 0.10 was considered, with the exception of the rs689466 polymorphism (GG vs AA) that presented an FPRP of 0.690, suggesting possible bias in this positive finding (data not shown).

Gene-environment Interaction Analysis

Upon a stratified analysis we observed, that with the exception of rs6439448 and rs1751051 polymorphisms in *SLCO2A1* and *ABCC4* gene, respectively all other variants appear to have a sex-dependent behavior particularly relevant in male carriers of GG genotype of *COX-2* rs689466 SNP (OR = 3.3; 95%CI:1.23–9.09, $P = 0.018$) and AA homozygous for the rs1426945 polymorphism in *HPGD* gene (OR = 0.38; 95%CI:0.20–0.74, $P = 0.004$), as reported in Table 3.

Furthermore, a nearly 6-fold increased risk was observed in ever-smokers carrying the GG genotype for the *COX-2* rs689466 polymorphism (95%CI:1.49–22.42, $P = 0.011$ vs OR = 0.63; 95%CI:0.13–3.08, $P = 0.56$ in never-smokers). In contrast, the *ABCC4* rs1751051 AA genotype seemed to lead to a higher susceptibility in individuals who never smoked (OR = 2.32; 95%CI:1.05–5.13, $P = 0.037$). The rs7616492 homozygous AA genotype of *SLCO2A1* gene played opposing roles when considering the interaction with BMI (OR = 0.06; 95%CI:0.01–0.69, $P = 0.023$ and OR = 2.18; 95%CI:1.00–4.77, $P = 0.051$ for individuals with BMI <25 and overweight (BMI≥25 kg/m^2), respectively).

Haplotype Analysis

Four common haplotypes were described for *COX-2* gene, as can be observed in Table 4. The most frequent haplotype, the AGT, was present in 52% of controls and used as the reference one. The block containing the rs689466 G allele, GGT, was associated with a 51% increased susceptibility consistent with the individual SNP analysis (95%CI:1.10–2.06, $P = 0.010$). Although we did not noticed any influence of rs5275 C allele in CRC risk independently, carriers of AGC haplotype had a 1.53–fold higher predisposition for CRC (95%CI:1.13–2.19, $P = 0.008$). The AGAC haplotype of *HPGD* gene was the most common (30%) out of the five blocks. An enhanced risk was observed for Individuals carrying the blocks, AGGC and ACGC, containing the rs1426945 G (OR = 1.70; 95%CI:1.22–2.37, $P = 0.002$ and OR = 1.60; 95%CI:1.04–2.44, $P = 0.031$, respectively). Coherently, the opposing rs1426945 AA genotype conferred a 40% risk reduction in the SNP analysis. The haplotype TAGAAC of *SLCO2A1* gene containing the decreased risk associated rs6439448 A allele and rs7616492 G allele led to a nearly 50% protection for CRC development compared with individuals carrying the TCAAAC reference block. The only common haplotype encompassing the rs1751051 A allele of *ABCC4* gene (AATTA) increased the susceptibility for CRC onset by over two-folds in contrast with the TATTA most frequent haplotype. No block contained the rs1751031 G allele.

Gene-gene Interaction Analysis

An exhaustive MDR analysis was carried-out to evaluate all possible combinations of rs689466, rs1346271, rs1426945, rs6439448, rs7616492, rs1751051 and rs1751031 polymorphisms proven to be associated with CRC onset in the individual SNP analysis. As shown in Table 5, we observed the highest CVC (10/10) and accuracy (0.6957) in the four-factor interaction model, which shows an interaction between rs1426945 *HPGD* polymorphism, rs6439448 *SLCO2A1* SNP and rs1751051 and rs1751031 polymorphisms in *ABCC4* gene. This gene-gene interaction was associated with a 5-fold increased risk for CRC development (95%CI:3.89–7.02, $P<0.0001$).

Discussion

Early screening and follow-up of individuals previously diagnosed with colorectal adenomas is the cornerstone of CRC prevention [3]. Nevertheless, the compliance rates in countries with implemented population-based CRC screening guidelines are far from the desirable for a successful impact in CRC incidence [4]. Although the regular use of NSAIDs has been consistently effective in the primary prevention of colorectal tumors its use is currently compromised by the onset of serious gastrointestinal side effects in average-risk population [24]. So, the challenge falls in the identification of biomarkers that could target higher-risk populations for colorectal screening and/or chemopreventive strategies.

In this case-control study we assessed the involvement of 51 tagSNPs in four genes (*COX-2/HPGD/SLCO2A1/ABCC4*) with key roles in PGE_2 pathway in CRC development. Our results indicate that seven genetic polymorphisms are implicated in colorectal carcinogenesis: the rs689466A>G in *COX-2*, the rs1346271G>C and rs1426945G>A in *HPGD*, the rs6439448C>A and rs7616492G>A in *SLCO2A1* and the rs1751051T>A and rs1751031A>G in *ABCC4* gene.

The rs689466A>G in *COX-2* gene had a synergetic effect in CRC oncogenesis that increased with allele dosage, further reinforcing its causative role in cancer development. The GG homozygous genotype enhanced the susceptibility for CRC onset by 2-fold and appeared to have a sex and smoking habits dependent behavior, with ever-smokers having a nearly 6-fold increased genetic predisposition for CRC. These data follow our previous observations from a preliminary study [19]. Furthermore, two haplotypes containing either the rs689466G (GGT) or the rs5275C alleles (AGC) led to a 50% increase on the risk for CRC. The lack of consistency observed between epidemiological studies addressing the rs689466A>G SNP in different ethnic backgrounds or cancer models appears to suggest that not only population stratification and lifestyle habits might modulate this polymorphism behavior but also its influence might be cell, tissue and pathological condition-dependent [19,25–29]. In fact, in a recently published study we reported that this polymorphism located at −1195 nucleotides upstream exon 1 increases *COX-2* transcriptional activity in two colon cancer cell lines [30]. This was also noticeable in human hepatoma cell lines [31] but antagonizes the increased promoter activity observed for the rs689466 A allele in gastric cancer cell lines [25]. COX-2 overexpression is suggested as one of the smoke-induced pathways involved in carcinogenesis [32,33]. Tobacco contains more than 60 identified carcinogens and even though some, such as, nicotine and benzo[a]pyrene, were shown to trigger COX-2 expression through *b*-adrenoceptors and ERK1/2 pathways, respectively, the patho-

Table 2. Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal cancer onset.

SNP rs	Cases n (%)	Controls n (%)	Univariate analysis			Multivariate analysis			
			OR	95%CI	P value	aOR	95%CI	P value	P bootstrap
COX-2									
rs689466									
AA	143 (58.8)	323 (68.4)	1.00	Reference	–	1.00	Reference	–	–
AG	85 (35.0)	133 (28.2)	**1.44**	**1.03–2.02**	**0.032**	**1.53**	**1.08–2.17**	**0.018**	**0.028**
GG	15 (6.2)	16 (3.4)	**2.12**	**1.02–4.40**	**0.040**	2.01	0.93–4.35	0.076	0.063
HPGD									
rs1346271									
GG	104 (42.4)	174 (36.2)	1.00	Reference	–	1.00	Reference	–	–
GC	97 (39.6)	246 (51.2)	**0.66**	**0.47–0.92**	**0.016**	**0.68**	**0.47–0.96**	**0.029**	**0.034**
CC	44 (18.0)	60 (12.5)	1.23	0.78–1.94	0.382	1.34	0.83–2.17	0.231	0.260
rs1426945									
GG	110 (44.7)	169 (35.3)	1.00	Reference	–	1.00	Reference	–	–
GA	108 (43.9)	233 (48.6)	**0.71**	**0.51–0.99**	**0.044**	0.70	0.50–1.00	0.050	0.055
AA	28 (11.4)	77 (16.1)	**0.56**	**0.34–0.92**	**0.021**	**0.56**	**0.34–0.93**	**0.026**	**0.035**
SLCO2A1									
rs6439448									
CC	174 (72.2)	319 (66.6)	1.00	Reference	–	1.00	Reference	–	–
CA	56 (23.2)	143 (29.9)	0.72	0.50–1.03	0.071	**0.68**	**0.47–0.99**	**0.047**	**0.039**
AA	11 (4.6)	17 (3.5)	1.19	0.54–2.59	0.668	0.93	0.39–2.20	0.869	0.851
rs7616492									
GG	89 (37.1)	202 (42.2)	1.00	Reference	–	1.00	Reference	–	–
GA	103 (42.9)	216 (45.1)	1.08	0.77–1.52	0.651	1.18	0.82–1.69	0.373	0.369
AA	48 (20.0)	61 (12.7)	**1.79**	**1.14–2.81**	**0.012**	2.05	**1.27–3.32**	**0.003**	**0.002**
ABCC4									
rs1751051									
TT	111 (46.2)	234 (48.8)	1.00	Reference	–	1.00	Reference	–	–
TA	91 (37.9)	202 (42.1)	0.95	0.68–1.33	0.763	1.06	0.74–1.50	0.764	0.758
AA	38 (15.8)	44 (9.2)	**1.82**	**1.12–2.97**	**0.016**	**1.76**	**1.04–2.95**	**0.034**	0.053
rs1751031									
AA	164 (66.9)	298 (62.2)	1.00	Reference	–	1.00	Reference	–	–
AG	66 (26.9)	166 (34.7)	0.72	0.51–1.02	0.063	**0.68**	**0.47–0.97**	**0.032**	**0.031**
GG	15 (6.1)	15 (3.1)	1.82	0.87–3.81	0.111	1.67	0.77–3.63	0.194	0.168

[a]Odds ratio (OR) adjusted for age (categorical variable, using the global median age of 60 years as cutoff); CI, confidence interval; bootstrap results are based in 1000 samples.
Statistical significant results are at bold.
doi:10.1371/journal.pone.0092000.t002

Table 3. Risk estimates for the involvement of polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes in colorectal cancer onset stratified by sex, smoking habits and body mass index.

Gene	n	aOR	95%CI	*P* value	$P_{bootstrap}$
COX-2					
rs689466 (AAvsGG)					
Sex					
Female	180	0.89	0.25–3.23	0.862	0.840
Male	313	3.34	1.23–9.09	**0.018**	**0.004**
Smoking habits					
Never-smokers	213	0.63	0.13–3.08	0.564	0.429
Ever-smokers*	142	5.77	1.49–22.42	**0.011**	**0.004**
BMI (kg/m^2)$^{\#}$					
$<$25	59	3.63	0.20–64.59	0.381	0.071
$\geq$25	182	2.41	0.72–8.07	0.154	0.123
HPGD					
rs1346271 (GGvsGC)					
Sex					
Female	214	0.42	0.23–0.78	**0.005**	**0.007**
Male	401	0.86	0.55–1.33	0.487	0.500
Smoking habits					
Never-smokers	271	0.88	0.51–1.51	0.644	0.613
Ever-smokers*	171	0.62	0.30–1.27	0.190	0.183
BMI (kg/m^2)$^{\#}$					
$<$25	69	0.57	0.19–1.69	0.312	0.312
$\geq$25	234	0.69	0.40–1.19	0.185	0.186
rs1426945 (GGvsAA)					
Sex					
Female	140	1.20	0.52–2.79	0.672	0.677
Male	211	0.38	0.20–0.74	**0.004**	**0.006**
Smoking habits					
Never-smokers	168	1.02	0.50–2.08	0.966	0.969
Ever-smokers*	101	0.83	0.31–2.21	0.705	0.730
BMI (kg/m^2)$^{\#}$					
$<$25	42	1.07	0.28–4.12	0.922	0.932
$\geq$25	135	1.29	0.59–2.86	0.525	0.563
SLCO2A1					
rs6439448 (CCvsCA)					
Sex					
Female	253	0.72	0.39–1.33	0.292	0.314
Male	433	0.66	0.41–1.07	0.094	0.082
Smoking habits					
Never-smokers	302	0.72	0.41–1.28	0.269	0.265
Ever-smokers*	185	0.64	0.29–1.41	0.272	0.277
BMI (kg/m^2)$^{\#}$					
$<$25	79	1.04	0.34–3.13	0.95	0.947
$\geq$25	258	0.75	0.42–1.34	0.34	0.312
rs7616492 (GGvsAA)					
Sex					
Female	135	1.60	0.72–3.58	0.250	0.254
Male	260	2.35	1.28–4.28	**0.005**	**0.008**
Smoking habits					
Never-smokers	163	1.48	0.64–3.27	0.37	0.382

Table 3. Cont.

Gene	n	aOR	95%CI	*P* value	$P_{bootstrap}$
Ever-smokers*	119	1.27	0.51–3.19	0.60	0.628
BMI (kg/m^2)$^{\#}$					
<25	50	0.06	0.006–0.69	**0.023**	**0.012**
≥25	142	2.18	1.00–4.77	0.051	0.050
ABCC4					
rs1751051 (TTvsAA)					
Sex					
Female	151	2.20	0.83–5.81	0.111	0.151
Male	269	1.70	0.91–3.16	0.096	0.100
Smoking habits					
Never-smokers	179	2.32	1.05–5.13	**0.037**	**0.033**
Ever-smokers*	114	1.26	0.44–3.57	0.665	0.681
BMI (kg/m^2)$^{\#}$					
<25	46	3.57	0.39–32.52	0.260	0.114
≥25	157	1.82	0.86–3.89	0.120	0.145
rs1751031 (AAvsAG)					
Sex					
Female	249	0.51	0.28–0.94	**0.030**	**0.031**
Male	437	0.78	0.49–1.22	0.269	0.269
Smoking habits					
Never-smokers	301	0.69	0.39–1.21	0.196	0.203
Ever-smokers*	193	0.76	0.38–1.52	0.439	0.454
BMI (kg/m^2)$^{\#}$					
<25	80	0.52	0.19–1.45	0.213	0.255
≥25	261	0.76	0.44–1.31	0.324	0.351

[a]Odds ratio (OR) adjusted for age (categorical variable, using the global median age of 60 years as cutoff); CI, confidence interval; BMI, body mass index; $^{\#}$Categorization based on the cutoff defined by the world Health Organization for overweight people;
*Never- and former-smokers pooled together.
Statistical significant results are at bold.
doi:10.1371/journal.pone.0092000.t003

genesis of smoking related CRC is still understudied [34]. Further functional studies are needed to elucidate the nature of this gene-environment interaction.

The rs5275T>C polymorphism, set at 8473 base pairs from exon 1 was previously associated with an increased risk for colorectal adenoma and here with a higher susceptibility for CRC in the context of the AGC haplotype (vs AGT) [18]. This T-to-C substitution in the 3′UTR was proven to contribute to COX-2 overexpression by disrupting the miR-542-3p:mRNA interaction and thus decreasing COX-2 mRNA decay [35].

As already mentioned, COX-2 has a predominant role in the synthesis of the pro-carcinogenic PGE_2 bioactive lipid and the main molecular target of NSAIDs. In fact Chan and colleagues [36] noticed that aspirin's preventive role was exclusively effective in the subgroup of colon cancers overexpressing COX-2 enzyme. So, the genetic variability in *COX-2* gene may help predict individuals at higher risk and expected to be exposed to higher levels of COX-2.

The expression and activity of 15-PGDH is repressed in colorectal cancer and Apc^{min} mouse adenomas, leading to a decrease in PGE_2 catabolism, local tissue accumulation of PGE_2 and resistance to Celecoxib chemoprevention in colon tumors [11,13].

We were not able to reproduce in our population the associations reported in previous studies [20,21,37]. This could be attributed to population stratification involving differences in genetic ancestry as the study developed by Hoeft and colleagues [20] involved participants from 10 different European countries or these variants could be in linkage disequilibrium with a causative SNP with a lower allele frequency (<15%) thus limiting our statistical power to detect a true association. Nevertheless, we observed for the first time that the rs1346271G>C and rs1426945G>A tagSNPs in *HPGD* gene were associated with a decrease risk for CRC development. Both of these genetic variations are located in the 5′UTR of *HPGD* gene altering the transcription factors binding sites as predicted by the SNPinfo software (www.snpinfo.niehs.nih.gov) that ultimately could lead to a differential expression of 15-PGDH. Remarkably, inherited mutations in *HPGD* gene are linked to the development of primary hypertrophic osteoarthropathy (PHO), thus reinforcing the impact that the genetic variability in *HPGD* might portray in disease development by disrupting the normal 15-HPGD levels or activity [38].

To the best of our knowledge this is the first study addressing the involvement of these specific genetic polymorphisms in *SLCO2A1* and *ABCC4* genes, coding for the PGT and MRP4 specific prostaglandin transporters, in disease development. The efflux-

Table 4. Haplotype frequencies between patients and controls and risk estimates for their involvement in colorectal cancer development.

Gene/Haplotype	% Cases	% Controls	aOR	95%CI	*P*
COX-2[€]					
A-G-T	44.9	52.4	1	Reference	–
G-G-T	21.9	17.3	1.51	1.10–2.06	**0.010**
A-G-C	18.3	13.4	1.57	1.13–2.19	**0.008**
A-C-C	10.3	15.1	0.82	0.56–1.20	0.310
*HPGD**					
A-G-A-C	23.8	30.5	1	Reference	–
A-G-G-C	25.4	17.8	1.70	1.22–2.37	**0.002**
A-C-G-T	17.9	20.1	1.12	0.77–1.62	0.550
A-C-G-C	12.8	10.8	1.60	1.04–2.44	**0.031**
G-G-A-C	6.4	8.0	1.05	0.57–1.92	0.880
SLCO2A1[£]					
T-C-A-A-A-C	25.7	26.1	1	Reference	–
T-A-G-A-A-C	8.3	12.7	0.54	0.33–0.82	**0.012**
C-C-G-A-A-C	9.6	10.6	0.86	0.54–1.36	0.520
T-C-G-A-A-C	6.8	9.0	0.75	0.44–1.25	0.270
T-C-G-A-G-C	6.5	8.7	0.68	0.40–1.15	0.150
ABCC4[¥]					
T-A-T-T-A	10.9	12.0	1	Reference	–
T-A-T-C-A	7.9	12.0	1.07	0.49–2.34	0.860
T-G-T-T-A	7.8	10.1	1.13	0.57–2.24	0.740
A-A-T-T-A	11.6	7.5	2.28	1.12–4.67	**0.024**
T-G-C-T-A	8.5	4.0	1.58	0.67–3.68	0.290
T-G-C-C-A	4.5	6.4	0.88	0.40–1.96	0.760

[a]Odds ratio (OR) adjusted for age (categorical variable, using the global median age of 60 years as cutoff); CI, confidence interval.
[€]SNPs order: rs689466-rs20417-rs5275.
*SNPs order: rs2612656-rs1346271-rs1426945-rs12500316.
[£]SNPs order: rs4241362-rs6439448-rs7616492-rs7625035-rs1131598-rs10935090.
[¥]SNPs order: rs1751051-rs2274403-rs1678405-rs1678396-rs1751031.
doi:10.1371/journal.pone.0092000.t004

dominated flow of PGE_2 in neoplastic tumors, due to an increased in COX-2 and MRP4 and repressed expression of 15-HPGD and PGT is associated with high levels of PGE_2 in the extracellular milieu culminating in the activation of a plethora of pathways that potentiate tumor development [8,11,16]. The rs6839448C>A and rs7617492G>A tagSNPs in *SLCO2A1* were implicated in colorectal carcinogenesis Furthermore, individuals carrying the haplotype containing both the A and G alleles of rs6839448 and rs7617492 tagSNPs (TAGAAC), respectively, had a nearly 50% protection for CRC. Although, the rs6439448 is not expected to be functional it tags two SNPs with predicted impact on PGT expression: the rs2370512T>A located in the 3′UTR that could affect the binding of microRNAs and stability of mRNA and the nonsynonymous rs34550074G>A SNP at codon 396 that codes for two different amino acids (Alanine>Threonine) with potential repercussion on protein structure and function.

Focusing on *ABCC4* gene, two tagSNPs influenced the genetic susceptibility for the development of CRC (rs1751051 and rs1751031), although none of the SNPs in the LD blocks tagged by these two SNPs could explain the altered risk for cancer development.

Table 5. MDR analysis for the colorectal cancer risk prediction.

Best model	CV accuracy	CV consistency	OR	95%CI	*P*
rs1346271, rs1426945	0.6113	10/10	2.53	1.91–3.35	<0.0001
rs1426945,rs6439448, rs1751031	0.6376	6/10	3.19	2.39–4.28	<0.0001
rs1426945,rs6439448, rs1751051, rs1751031	0.6957	10/10	5.23	3.89–7.02	<0.0001

MDR, multifactor dimensionality reduction; CV, cross-validation; OR, odds ratio; CI, confidence interval.
doi:10.1371/journal.pone.0092000.t005

Common diseases have proven to be much more challenging to understand, as they are thought to arise due to the synergetic effect of many different susceptibility DNA variants interacting with environmental factors. Although, we have noticed some interactions between the aforementioned tagSNPs and demographic/lifestyle habits, the lack of complete characterization of the study population, decreased the statistical power and the scarcity of studies inquiring the influence of those environmental factors specifically in these key players in PGE_2 pathway have compromised the interpretation of those associations. Furthermore, we used the data-mining analytical approach, MDR, to enhance the likelihood of identifying gene-gene interactions and a strong interaction between four SNPs in *HPGD*, *SCO2A1* and *ABCC4* genes reinforcing the data from single–locus analysis and lending further support to the involvement of genetic susceptibility biomarkers in colorectal carcinogenesis.

There are a few limitations that should be considered. First, this study has a case-control design, so we could not rule out selection bias, although if this was the case our results would tend to have strong associations; or recall bias that could decrease the accuracy of collected data. Second, our sample size allowed us to detect strong associations in the overall analysis for frequent polymorphisms, so we cannot exclude the influence of rarer SNPs or with more modest influences in the *PTGS2/HPGD/SLCO2A1/ABCC4* genes in CRC development. Furthermore, and although we employed statistical strategies to assess the robustness of associations, namely the use of bootstrap resampling, an independent and larger data set is needed to corroborate our findings and allow a more comprehensive understanding of the gene-environment interactions.

In conclusion, we observed that seven tagSNPs in key genes regulating the procarcinogenic-PGE_2 levels in tumor microenvironment were implicated in CRC development. Particularly, the *COX-2* rs689466GG genotype in ever-smokers and a gene-gene interaction involving the rs1426945 *HPGD* polymorphism, rs6439448 *SLCO2A1* SNP and rs1751051 and rs1751031 polymorphisms in *ABCC4* gene. A clearer understanding on CRC etiology through the identification of risk biomarkers might allow a better definition of risk models that are more likely to benefit from targeted preventive strategies.

Supporting Information

Table S1 Genetic polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes characterization and quality control results.
(DOCX)

Table S2 Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal cancer onset.
(DOCX)

Author Contributions

Conceived and designed the experiments: CP RM MDR. Performed the experiments: CP SQ HS AG PPN CB LMD MDR. Analyzed the data: CP RM MDR. Wrote the paper: CP RM MDR.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, et al. (2010) GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer; Available from: http://globocan.iarc.fr.
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, et al. (2011) Global Cancer Statistics. CA Cancer J Clin 61: 69–90.
3. Edwards BK, Ward E, Kohler BA, Eheman C, Zauber AG, et al. (2010) Annual report to the nation on the status of cancer, 1975–2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. Cancer 116: 544–573.
4. Gimeno Garcia AZ (2012) Factors influencing colorectal cancer screening participation. Gastroenterol Res Pract 2012: 483417.
5. Flossmann E, Rothwell PM (2007) Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet 369: 1603–1613.
6. Algra AM, Rothwell PM (2012) Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomized trials. Lancet Oncol 13(5): 518–527.
7. Vane JR, Blotting RM (1998) Mechanism of action of nonsteroidal anti-inflammatory drugs. Am J Med 104(3A): 2S–8S.
8. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, et al. (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107: 1183–1188.
9. Wang D, Mann JR, Dubois RN (2005) The role of prostaglandins and other eicosanoids in the gastrointestinal tract. Gastroenterology 128: 1445–1461.
10. Tai HH, Ensor CM, Tong M, Zhou H, Yan F (2002) Prostaglandin catabolizing enzymes. Prostaglandins Other Lipid Mediat 68–69: 483–493.
11. Backlund MG, Mann JR, Holla VR, Buchanan FG, Tai HH, et al. (2005) 15-Hydroxyprostaglandin dehydrogenase is down-regulated in colorectal cancer. J Biol Chem 280: 3217–3223.
12. Myung SJ, Rerko RM, Yan M, Buchanan FG, Tai HH, et al. (2006) 15-Hydroxyprostaglandin dehydrogenase is an in vivo suppressor of colon tumorigenesis. Proc Natl Acad Sci 103(32): 12098–12102.
13. Yan M, Myung SJ, Fink SP, Lawrence E, Lutterbaugh J, et al. (2009) 15-Hydroxyprostaglandin dehydrogenase inactivation as a mechanism of resistance to Celecoxib chemoprevention of colon tumors. Proc Natl Acad Sci 106(23): 9409–9413.
14. Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, et al. (2003) The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci 100(16): 9244–9249.
15. Schuster VL (2002). Prostaglandin transport. Prostaglandins Other Lipid Mediat 68–69: 633–647.
16. Holla VR, Backlund MG, Yang P, Newman RA, DuBois RN (2008) Regulation of prostaglandin transporters in colorectal neoplasia. Cancer Prev Res 1(2): 93–99.
17. Lynch HT, de la Chapelle A (2003) Hereditary colorectal cancer. N Engl J Med 348(10): 919–932.
18. Pereira C, Medeiros R, Dinis-Ribeiro M (2009) Cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis: are conclusive results available? Eur J Gastroenterol Hepatol 21: 76–91.
19. Pereira C, Pimentel-Nunes P, Brandão C, Moreira-Dias L, Medeiros R, et al. (2010) COX-2 polymorphisms and colorectal cancer risk: a strategy for chemoprevention. Eur J Gastroenterol Hepatol 22(5): 607–663.
20. Hoeft B, Linseisen J, Beckmann L, Muller-Decker K, Canzian F, et al. (2010) Polymorphisms in fatty acid metabolism-related genes are associated with colorectal cancer risk. Carcinogenesis 31(3): 466–472.
21. Thompson CL, Fink SP, Lutterbaugh JD, Elston RC, Veigl ML, et al. (2013) Genetic variation in 15-Hydroxyprostaglandin dehydrogenase and colon cancer susceptibility. PLoS One 8(5): e64122.
22. Abulí A, Fernández-Rozadilla C, Giráldez MD, Munõz J, Gonzalo V, et al. (2011) A two-phase case–control study for colorectal cancer genetic susceptibility: candidate genes from chromosomal regions 9q22 and 3q22. BJC 105: 870–875.
23. Wacholder S, Chanock S, Garcia-Closas M, Rothman N (2004) Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. J Natl Cancer Inst 96: 434–442.
24. Rodríguez LA, Tolosa LB (2009) Risk of upper gastrointestinal complications among users of Traditional NSAIDs and COXIBs in the general population. Gastroenterology 132(2): 4498–4506.
25. Liu F, Pan K, Zhang X, Zhang Y, Zhang L, et al. (2006) Genetic variants in cyclooxygenase-2: Expression and risk of gastric cancer and its precursors in a Chinese population. Gastroenterology 130: 1975–1984.
26. Zhang X, Miao X, Tan W, Ning B, Liu Z, et al. (2005) Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer. Gastroenterology 129: 565–576.
27. Tan W, Wu J, Zhang X, Guo Y, Liu J, et al. (2007) Associations of functional polymorphisms in cyclooxygenase-2 and platelet 12-lipoxygenase with risk of occurrence and advanced disease status of colorectal cancer. Carcinogenesis 28: 1197–1201.
28. Ueda N, Maehara Y, Tajima O, Tabata S, Wakabayashi K, et al. (2008) Genetic polymorphisms of cyclooxygenase-2 and colorectal adenoma risk: The Self Defense Forces Health Study. Cancer Sci 99: 576–581.
29. Peters WH, te Morsche RH, Roelofs HM, Mathus-Vliegen EM, Berhout M, et al. (2009) COX-2 polymorphisms in patients with familial adenomatous polyposis. Oncol Res 17: 347–351.

30. Pereira C, Sousa H, Silva J, Brandão C, Elgueta-Karstegl C, et al. (2013) The −1195G allele increases the transcriptional activity of cyclooxygenase-2 gene (COX-2) in colon cancer cell lines. Mol Carcinog. doi: 10.1002/mc.22049.
31. Sakaki M, Makino R, Hiroishi K, Ueda K, Eguchi J, et al. (2010) Cyclooxygenase-2 gene promoter polymorphisms affect susceptibility to hepatitis C virus infection and disease progression. Hepatol Res 40: 1219–1226.
32. Martey CA, Pollock SJ, Turner CK, O'Reilly KM, Baglole CJ, et al. (2004) Cigarette smoke induces cyclooxygenase-2 and microsomal prostaglandin E2 synthase in human lung fibroblasts: implications for lung inflammation and cancer. Am J Physiol Lung Cell Mol Physiol 287: L981–L991.
33. Wong HP, Yu L, Lam EK, Tai EK, Wu WK, et al. (2007) Nicotine promotes colon tumor growth and angiogenesis through beta-adrenergic activation. Toxicol Sci 97: 279–287.
34. Yan Z, Subbaramaiah K, Camilli T, Zhang F, Tanabe T, et al. (2000) Benzo[a]pyrene induces the transcription of cyclooxygenase-2 in vascular smooth muscle cells. Evidence for the involvement of extracellular signal-regulated kinase and NF-kappaB. J Biol Chem 275: 4949–4955.
35. Moore AE, Young LE, Dixon DA (2012) A common single-nucleotide polymorphism in cyclooxygenase-2 disrupts micro-RNA-mediated regulation. Oncogene 31(12): 1592–1598.
36. Chan AT, Ogino S, Fuchs CS (2007) Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. N Engl J Med 356: 2131–2142.
37. Frank B, Hoeft B, Hoffmeister M, Linseisen J, Breitling LP, et al. (2010) Association of hydroxyprostaglandin dehydrogenase 15-(NAD) (*HPGD*) variants and colorectal cancer risk. Carcinogenesis 32(2): 190–196.
38. Sinibaldi L, Harifi G, Bottillo I, Iannicelli M, El Hassani S, et al. (2010) A novel homozygous splice site mutation in the HPGD gene causes mild primary hypertrophic osteoarthropathy. Clin Exp Rheumatol 28(2): 153–157.

Table S1. Characterization of genetic polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes and quality control results

Gene	tagSNP	Other SNPs on the block	Genotype call rate	Genotype concordance rate	HWE	Passed quality check?
COX-2	rs689466	Candidate gene	0.98	0.97	0.901	Yes
	rs20417	Candidate gene	0.98	0.96	0.998	Yes
	rs5275	Candidate gene	0.97	1.00	0.999	Yes
HPGD	rs2555639	Candidate gene	0.98	1.00	0.989	Yes
	rs1346271	singleton	1.00	1.00	0.167	Yes
	rs2555632	rs3101255	1.00	1.00	0.681	Yes
	rs2303520	rs13127058	0.99	1.00	0.633	Yes
	rs1863642	rs2612659	0.99	1.00	0.474	Yes
	rs1426945	rs3756273	1.00	0.97	0.976	Yes
	rs12500316	rs1863641	0.99	1.00	0.508	Yes
		rs11722919				
	rs8752	rs1426947	1.00	1.00	0.948	Yes
		rs2612658				
		rs11133041				
		rs11724251				
	rs2612656	rs1816204	0.92	0.96	0.917	Yes
		rs3857075				
SLCO2A1	rs4241362	rs4241361	0.99	1.00	0.756	Yes
		rs4634113				
		rs6804798				
		rs9828294				
		rs9855403				
		rs9874493				
		rs9882333				
		rs11720811				
	rs764392	rs4327389	0.98	1.00	0.550	Yes
		rs4854777				
		rs5013525				
		rs7646298				
		rs7646473				
		rs12695600				
	rs6439448	rs2370512	0.99	0.97	0.979	Yes
		rs3923835				
		rs3923835				
		rs4854768				
		rs4854769				
		rs34550074				
	rs9821091	rs7630191	1.00	1.00	0.651	Yes
		rs9841380				
		rs6439450				
		rs7617777				
		rs9834727				
	rs9820625	rs9836830	1.00	1.00	0.948	Yes
		rs9917636				
		rs11709172				
		rs13083175				
	rs9834412	rs4854785	0.98	1.00	0.951	Yes
		rs13067921				
	rs4241365	rs7653639	0.99	1.00	0.923	Yes
	rs4331673	rs11720843	1.00	1.00	0.994	Yes
	rs4854784	rs7636169	0.98	0.97	0.871	Yes
	rs7340717	rs7340718	0.98	1.00	0.600	Yes
	rs7616492	rs10935089	0.99	1.00	0.938	Yes
	rs7625035	rs9822027	1.00	1.00	0.455	Yes
	rs1131598	Singleton	1.00	1.00	0.441	Yes
	rs10935090	Singleton	0.99	1.00	0.815	Yes
	rs11915399	Singleton	0.99	1.00	0.998	Yes
ABCC4	rs4148422	rs17300935	1.00	0.95	0.006	No
	rs9524821	[rs9516532]	0.99	1.00	0.604	Yes
	rs3782958	rs4148515	0.99	1.00	0.931	Yes
		rs10508023				
	rs869951	rs871052	1.00	1.00	0.854	Yes
		rs8001444				
	rs4771912	rs7981095	0.99	0.97	0.936	Yes
	rs4148421	rs9524864	0.98	1.00	0.998	Yes
		rs9524873				
		rs10508017				

Table S1. Characterization of genetic polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes and quality control results

Gene	tagSNP	Other SNPs on the block	Genotype call rate	Genotype concordance rate	HWE	Passed quality check?
ABCC4	rs8002180	rs4148424	1.00	1.00	0.694	Yes
		rs4771910				
		rs7317112				
		rs7322318				
		rs8001475				
		rs9584288				
		rs9590228				
	rs9590222	rs12100301	0.95	1.00	<0.001	No
	rs2127295	rs2698243	0.99	0.97	0.657	Yes
		rs1564355				
		rs1617785				
		rs1630807				
		rs1678363				
		rs1678394				
		rs1729748				
		rs2766481				
		rs3825415				
		rs6650282				
	rs1751051	[rs1751050]	0.99	1.00	0.999	Yes
	rs9590220	rs9590216	1.00	0.96	0.018	No
		rs17235152				
	rs2892715	rs9561814	1.00	1.00	0.473	Yes
	rs2892713	rs12865305	1.00	1.00	0.313	Yes
	rs4612933	rs899494	1.00	1.00	0.936	Yes
		rs899495				
		rs899496				
		rs1678403				
		rs1824911				
		rs1824913				
		rs1926657				
		rs3782965				
		rs4148465				
		rs4148469				
		rs4303338				
		rs4334136				
		rs4505186				
		rs4773854				
		rs4773855				
		rs7325019				
		rs7333234				
		rs7335147				
		rs7983336				
		rs7987653				
		rs7988494				
		rs9524831				
		rs9524833				
		rs9524845				
		rs9524856				
		rs12870204				
	rs4148437	rs9556466	99.3	1.00	0.665	Yes
		rs2892716				
		rs4148436				
		rs4148446				
		rs10508018				
	rs12867485	rs9561811	0	-	-	No
		rs17189481				
	rs1611822	rs1751015	1.00	1.00	0.969	Yes
	rs1678386	rs9516530	1.00	1.00	0.818	Yes
	rs2274403	rs3864997	1.00	0.97	0.862	Yes
		rs4148481				
	rs17268122	rs17268163	0.93	0.96	0.027	No
	rs1751027	rs1564351	1.00	1.00	0.396	Yes
		rs4148487				
		rs17189390				
		rs17268170				
	rs4148476	rs4773843	1.00	1.00	0.280	Yes
		rs9524822				
	rs1678374	rs1751025	0.99	1.00	0.964	Yes

Table S1. Characterization of genetic polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes and quality control results

Gene	tagSNP	Other SNPs on the block	Genotype call rate	Genotype concordance rate	HWE	Passed quality check?
ABCC4	rs1678405	rs2793821	0.99	1.00	0.722	Yes
		rs6492768				
		rs7330933				
	rs1678396	rs2766482	1.00	1.00	0.537	Yes
	rs1628382	rs4148527	0.27	1.00	-	No
		rs8001657				
		rs12584534				
	rs1678354	rs1751059	0	-	-	No
	rs1751031	rs931111	1.00	1.00	0.372	Yes
		rs1189444				
		rs1189451				
		rs1189452				
		rs1729747				
		rs2619312				
		rs5016378				
	rs7993878	rs9302040	1.00	0.97	0.346	Yes
		rs9302042				
		rs9302043				
		rs9556455				
		rs9561768				
		rs9561769				
		rs9590168				
		rs10219913				
	rs6492763	rs10508024	0.99	0.97	0.520	Yes
	rs3742106	rs4148544	0.99	1.00	0.987	Yes
		rs4148549				
		rs4148551				
		rs7330196				
		rs9302039				
		rs9524769				

Table S2. Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal cancer onset

SNPs rs	Cases n (%)	Controls n (%)	OR	95%CI	*P* value	aOR	95%CI	*P* value
COX-2								
rs689466								
AA	143 (58.8)	323 (68.4)	1.00	Reference	-	1.00	Reference	-
AG	85 (35.0)	133 (28.2)	1.44	1.03-2.02	0.032	1.53	1.08-2.17	0.018
GG	15 (6.2)	16 (3.4)	2.12	1.02-4.40	0.040	2.02	0.93-4.39	0.076
rs20417								
GG	179 (74.9)	328 (69.3)	1.00	Reference	-	1.00	Reference	-
GC	55 (23.0)	132 (27.9)	0.76	0.53-1.10	0.145	0.80	0.55-1.16	0.390
CC	5 (2.1)	13 (2.7)	0.71	0.25-2.01	0.511	0,64	0.22-1.90	
s5275								
TT	122 (50.8)	235 (50.9)	1.00	Reference	-	1.00	Reference	-
TC	89 (37.1)	189 (40.9)	0.91	0.65-1.27	0.567	0.93	0.66-1.32	0.390
CC	29 (12.1)	38 (8.2)	1.47	0.86-2.50	0.153	1.39	0.80-2.41	
HPGD								
rs2555629								
TT	111 (46.1)	216 (45.6)	1.00	Reference	-	1.00	Reference	-
TC	101 (41.9)	209 (44.1)	0.94	0.68-1.31	0.715	0.99	0.70-1.40	0.960
CC	29 (12.0)	49 (10.3)	1.15	0.69-1.92	0.589	1.07	0.63-1.83	
rs2612656								
AA	160 (72.7)	295 (65.6)	1.00	Reference	-	1.00	Reference	-
AG	47 (21.4)	137 (30.4)	0.63	0.43-0.93	0.019	0.71	0.48-1.05	0.110
GG	13 (5.9)	18 (4.0)	1.33	0.64-2.79	0.446	1.48	0.69-3.18	
rs8752								
TT	91 (37.1)	197 (41.2)	1.00	reference	-	1.00	Reference	-
TC	112 (45.7)	219 (45.8)	1.11	0.79-1.55	0.553	1.16	0.82-1.65	0.398
CC	42 (17.1)	62 (13.0)	1.47	0.92-2.33	0.105	1.61	0.98-2.62	0.059
rs1346271								
GG	104 (42.4)	174 (36.2)	1.00	reference	-	1.00	Reference	-
GC	97 (39.6)	246 (51.2)	0.66	0.47-0.92	0.016	0.68	0.47-0.96	0.029
CC	44 (18.0)	60 (12.5)	1.23	0.78-1.94	0.382	1.34	0.83-2.17	0.231
rs2555632								
TT	143 (58.4)	284 (59.3)	1.00	reference	-	1.00	Reference	-
TC	88 (35.9)	174 (36.3)	1.00	0.72-1.39	0.979	1.14	0.81-1.60	0.470
CC	14 (5.7)	21 (4.4)	1.32	0.65-2.68	0.434	1.44	0.69-3.00	0.331
rs2303520								
GG	167 (69.3)	342 (71.4)	1.00	reference	-	1.00	Reference	-
GA	66 (27.4)	123 (25.7)	1.10	0.77-1.56	0.599	1.09	0.76-1.58	0.641
AA	8 (3.3)	14 (2.9)	1.17	0.48-2.84	0.728	1.30	0.52-3.28	0.576
rs1863642								
GG	126 (52.3)	231 (48.1)	1.00	reference	-	1.00	Reference	-
GT	96 (39.8)	212 (44.2)	0.83	0.60-1.15	0.261	0.81	0.58-1.14	0.233
TT	19 (7.9)	37 (7.7)	0.94	0.52-1.71	0.842	0.94	0.51-1.76	0.854
rs1426945								
GG	110 (44.7)	169 (35.3)	1.00	reference	-	1.00	Reference	-
GA	108 (43.9)	233 (48.6)	0.71	0.51-0.99	0.044	0.70	0.50-1.00	0.050
AA	28 (11.4)	77 (16.1)	0.56	0.34-0.92	0.020	0.56	0.34-0.93	0.026
rs12500316								
CC	150 (62.0)	262 (54.7)	1.00	reference	-	1.00	Reference	-
CT	78 (32.2)	191 (39.9)	0.71	0.51-0.99	0.045	0.73	0.52-1.03	0.071
TT	14 (5.8)	26 (5.4)	0.94	0.48-1.86	0.860	1.01	0.50-2.08	0.972
SLCO2A1								
rs4241362								
TT	152 (63.3)	333 (69.5)	1.00	reference	-	1.00	Reference	-
TC	73 (30.4)	130 (27.1)	1.23	0.87-1.74	0.239	1.14	0.79-1.63	0.487
CC	15 (6.2)	16 (3.3)	2.05	0.99-4.26	0.049	1.82	0.84-3.94	0.130
rs7646392								
CC	92 (38.8)	175 (36.5)	1.00	reference	-	1.00	Reference	-
CT	97 (40.9)	220 (45.8)	0.84	0.59-1.19	0.321	0.97	0.67-1.40	0.869
TT	48 (20.3)	85 (17.7)	1.07	0.70-1.66	0.747	1.28	0.81-2.04	0.286
rs6439448								
CC	174 (72.2)	320 (66.7)	1.00	reference	-	1.00	Reference	-
CA	56 (23.2)	143 (29.8)	0.72	0.50-1.03	0.073	0.68	0.47-1.00	0.047
AA	11 (4.6)	17 (3.5)	1.19	0.54-2.60	0.662	0.93	0.39-2.20	0.869

Table S2. Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal cancer onset

SNPs rs	Cases n (%)	Controls n (%)	OR	95%CI	*P* value	aOR	95%CI	*P* value
rs9821091								
GG	110 (44.5)	180 (37.6)	1.00	reference	-	1.00	Reference	-
GA	105 (42.5)	235 (49.1)	0.73	0.52-1.02	0.063	0.79	0.56-1.12	0.181
AA	32 (13.0)	64 (13.4)	0.82	0.50-1.33	0.418	0.86	0.52-1.43	0.561
rs9820625								
AA	77 (31.3)	141 (29.4)	1.00	reference	-	1.00	Reference	-
AC	110 (44.7)	232 (48.3)	0.87	0.61-1.24	0.440	1.00	0.69-1.46	0.998
CC	59 (24.0)	107 (22.3)	1.01	0.66-1.54	0.964	1.14	0.73-1.78	0.574
rs9834412								
CC	140 (59.6)	270 (56.1)	1.00	reference	-	1.00	Reference	-
CA	76 (32.3)	179 (37.2)	0.82	0.58-1.15	0.245	0.78	0.55-1.11	0.170
AA	19 (8.1)	32 (6.7)	1.14	0.63-2.09	0.660	1.13	0.60-2.11	0.707
rs4241365								
TT	156 (64.5)	282 (58.9)	1.00	reference	-	1.00	Reference	-
TC	72 (29.8)	169 (35.3)	0.77	0.55-1.08	0.130	0.82	0.57-1.16	0.255
CC	14 (5.8)	28 (5.8)	0.90	0.46-1.77	0.768	1.05	0.52-2.13	0.888
rs4331673								
CC	153 (62.4)	332 (69.2)	1.00	reference	-	1.00	Reference	-
CA	84 (34.3)	134 (27.9)	1.36	0.98-1.90	0.070	1.30	0.92-1.84	0.145
AA	8 (3.3)	14 (2.9)	1.24	0.51-3.02	0.635	1.22	0.48-3.06	0.679
rs4854784								
GG	106 (45.1)	215 (44.9)	1.00	reference	-	1.00	Reference	-
GA	97 (41.3)	208 (43.4)	0.95	0.68-1.32	0.745	1.00	0.70-1.41	0.989
AA	32 (13.6)	56 (11.7)	1.16	0.71-1.90	0.557	1.35	0.80-2.28	0.255
rs7340717								
GG	105 (44.5)	204 (42.5)	1.00	reference	-	1.00	Reference	-
GT	90 (38.1)	210 (43.8)	0.83	0.59-1.17	0.293	0.83	0.58-1.18	0.299
TT	41 (17.4)	66 (13.8)	1.21	0.76-1.90	0.418	0.99	0.61-1.60	0.952
rs7616492								
GG	89 (37.1)	202 (42.1)	1.00	reference	-	1.00	Reference	-
GA	103 (42.9)	216 (45.0)	1.08	0.77-1.52	0.651	1.18	0.82-1.69	0.373
AA	48 (20.0)	62 (12.9)	1.76	1.12-2.76	0.014	2.05	1.27-3.32	0.003
rs7625035								
AA	139 (57.0)	278 (57.9)	1.00	reference	-	1.00	Reference	-
AG	85 (34.8)	181 (37.7)	0.94	0.68-1.30	0.708	0.92	0.65-1.29	0.619
GG	20 (8.2)	21 (4.4)	1.91	1.00-3.63	0.047	1.60	0.82-3.12	0.168
rs1131598								
AA	136 (55.7)	274 (5.1)	1.00	reference	-	1.00	Reference	-
AG	88 (36.1)	184 (38.3)	0.96	0.70-1.34	0.824	0.90	0.64-1.27	0.563
GG	20 (8.2)	22 (4.6)	1.83	0.97-3.47	0.061	1.79	0.92-3.50	0.087
rs10935090								
CC	180 (74.4)	382 (79.7)	1.00	reference	-	1.00	Reference	-
CT	54 (22.3)	90 (18.8)	1.27	0.87-1.86	0.213	1.28	0.86-1.90	0.222
TT	8 (3.3)	7 (1.5)	2.42	0.87-6.79	0.082	2.33	0.80-6.83	0.123
rs11915399								
CC	173 (70.0)	328 (68.5)	1.00	reference	-	1.00	Reference	-
CT	66 (26.7)	137 (28.6)	0.91	0.65-1.29	0.608	0.99	0.69-1.42	0.953
TT	8 (3.2)	14 (2.9)	1.08	0.45-2.63	0.860	1.11	0.44-2.79	0.832
ABCC4								
rs9524821								
GG	92 (37.9)	205 (42.8)	1.00	reference	-	1.00	Reference	-
GA	116 (47.7)	209 (43.6)	1.24	0.88-1.73	0.213	1.30	0.91-1.84	0.145
AA	35 (14.4)	65 (13.6)	1.20	0.74-1.94	0.456	1.18	0.70-1.98	0.534
rs3782958								
GG	173 (70.9)	336 (70.1)	1.00	reference	-	1.00	Reference	-
GC	62 (25.4)	129 (26.9)	0.93	0.66-1.33	0.700	0.89	0.62-1.29	0.894
CC	9 (3.7)	14 (2.9)	1.25	0.53-2.94	0.611	1.15	0.48-2.78	0.755
rs869951								
GG	101 (41.1)	171 (35.6)	1.00	reference	-	1.00	Reference	-
GC	103 (41.9)	226 (47.1)	0.77	0.55-1.08	0.133	0.75	0.53-1.07	0.120
CC	42 (17.1)	83 (17.3)	0.86	0.55-1.34	0.496	0.88	0.56-1.40	0.600
rs4771912								
AA	189 (79.4)	359 (74.6)	1.00	reference	-	1.00	Reference	-
AG	48 (20.2)	112 (23.3)	0.81	0.56-1.19	0.290	0.80	0.54-1.19	0.275
GG	1 (0.4)	10 (2.1)	0.19	0.02-1.50	0.078	0.17	0.02-1.38	0.097

Table S2. Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal cancer onset

SNPs rs	Cases n (%)	Controls n (%)	OR	95%CI	*P* value	aOR	95%CI	*P* value
rs4148421								
GG	71 (30.1)	134 (28.0)	1.00	reference	-	1.00	Reference	-
GA	111 (47.0)	238 (49.7)	0.88	0.61-1.27	0.494	0.84	0.57-1.24	0.381
AA	54 (22.9)	107 (22.3)	0.95	0.62-1.47	0.827	1.02	0.64-1.61	0.939
rs8002180								
TT	124 (50.6)	248 (51.8)	1.00	reference	-	1.00	Reference	-
TC	97 (39.6)	188 (39.2)	1.03	0.74-1.43	0.850	0.98	0.70-1.38	0.909
CC	24 (9.8)	43 (9.0)	1.12	0.65-1.92	0.692	1.03	0.58-1.84	0.913
rs2127295								
GG	70 (28.8)	137 (28.7)	1.00	reference	-	1.00	Reference	-
GA	130 (53.5)	247 (51.7)	1.03	0.72-1.47	0.871	1.00	0.69-1.45	0.998
AA	43 (17.7)	94 (19.7)	0.90	0.56-1.42	0.639	0.85	0.52-1.38	0.508
rs1751051								
TT	112 (46.5)	234 (48.8)	1.00	reference	-	1.00	Reference	-
TA	91 (37.8)	202 (42.1)	0.94	0.67-1.32	0.723	1.06	0.74-1.50	0.764
AA	38 (15.8)	44 (9.2)	1.80	1.11-2.94	0.017	1.76	1.04-2.95	0.034
rs2892715								
GG	103 (42.0)	173 (36.0)	1.00	reference	-	1.00	Reference	-
GA	100 (40.8)	220 (45.7)	0.76	0.54-1.07	0.119	0.74	0.52-1.06	0.102
AA	42 (17.1)	88 (18.3)	0.80	0.52-1.25	0.326	0.82	0.52-1.30	0.406
rs2892713								
CC	169 (68.7)	337 (70.4)	1.00	reference	-	1.00	Reference	-
CT	66 (26.8)	124 (25.9)	1.06	0.75-1.51	0.740	1.13	0.78-1.63	0.514
TT	11 (4.5)	18 (3.8)	1.22	0.56-2.64	0.615	1.25	0.56-2.81	0.582
rs4612933								
CC	160 (65.8)	315 (65.5)	1.00	reference	-	1.00	Reference	-
CT	71 (29.2)	148 (30.8)	0.94	0.67-1.33	0.743	0.95	0.66-1.36	0.777
TT	12 (4.9)	18 (3.7)	1.31	0.62-2.79	0.479	1.31	0.59-2.90	0.510
rs4148437								
TT	107 (44.0)	194 (40.4)	1.00	reference	-	1.00	Reference	-
TC	104 (42.8)	215 (44.8)	0.88	0.63-1.22	0.439	0.80	0.56-1-13	0.201
CC	32 (13.2)	71 (14.8)	0.82	0.51-1.32	0.409	0.81	0.50-1.33	0.406
rs1611822								
CC	77 (31.4)	182 (37.9)	1.00	reference	-	1.00	Reference	-
CT	126 (51.4)	225 (46.9)	1.32	0.94-1.87	0.110	1.44	0.89-2.07	0.060
TT	42 (17.1)	73 (15.2)	1.36	0.86-2.16	0.193	1.38	0.85-2.22	0.190
rs1678386								
AA	124 (50.6)	243 (50.6)	1.00	reference	-	1.00	Reference	-
AC	90 (36.7)	193 (40.2)	0.91	0.66-1.27	0.593	0.90	0.64-1.27	0.541
CC	31 (12.7)	44 (9.2)	1.38	0.83-2.30	0.212	1.33	0.77-2.30	0.304
rs2274403								
AA	74 (30.2)	120 (25.0)	1.00	reference	-	1.00	Reference	-
AG	122 (49.8)	234 (48.8)	0.84	0.59-1.22	0.365	0.90	0.61-1.32	0.594
GG	49 (20.0)	126 (26.2)	0.63	0.41-0.98	0.039	0.66	0.42-1.03	0.067
rs1751027								
AA	202 (82.1)	402 (83.8)	1.00	reference	-	1.00	Reference	-
AG	40 (16.3)	77 (16.0)	1.03	0.68-1.57	0.876	1.13	0.73-1.75	0.588
GG	4 (1.6)	1 (0.2)	7.96	0.88-71.69	0.047	6.11	0.65-57.40	0.113
rs4148476								
TT	181 (74.2)	339 (70.6)	1.00	reference	-	1.00	Reference	-
TG	56 (23.0)	123 (25.6)	0.85	0.59-1.23	0.390	0.89	0.61-1.31	0.559
GG	7 (2.9)	18 (3.8)	0.73	0.30-1.78	0.484	0.76	0.30-1.92	0.565
rs1678374								
TT	90 (37.0)	164 (34.2)	1.00	reference	-	1.00	Reference	-
TC	118 (48.6)	235 (49.1)	0.92	0.65-1.28	0.608	1.02	0.71-1.45	0.931
CC	35 (14.4)	80 (16.7)	0.80	0.50-1.28	0.347	0.77	0.48-1.26	0.303
rs1678405								
TT	116 (48.5)	199 (41.4)	1.00	reference	-	1.00	Reference	-
TC	103 (43.1)	227 (47.2)	0.78	0.56-1.08	0.132	0.75	0.54-1.06	0.104
CC	20 (8.4)	55 (11.4)	0.62	0.36-1.09	0.097	0.56	0.31-1.01	0.056
rs1678396								
TT	94 (38.2)	147 (30.6)	1.00	reference	-	1.00	Reference	-
TC	105 (42.7)	248 (51.7)	0.66	0.47-0.94	0.019	0.73	0.50-1.04	0.084
CC	47 (19.1)	85 (17.7)	0.86	0.56-1.34	0.518	0.88	0.56-1.40	0.590

Table S2. Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal cancer onset

SNPs rs	Cases n (%)	Controls n (%)	OR	95%CI	*P* value	aOR	95%CI	*P* value
rs1751031								
AA	165 (67.1)	299 (62.3)	1.00	reference	-	1.00	Reference	-
AG	66 (26.8)	166 (34.6)	0.72	0.51-1.02	0.060	0.68	0.47-0.97	0.032
GG	15 (6.1)	15 (3.1)	1.81	0.86-3.80	0.111	1.67	0.77-3.63	0.194
rs7993878								
GG	190 (77.2)	361 (75.1)	1.00	reference	-	1.00	Reference	-
GA	47 (19.1)	107 (22.2)	0.84	0.57-1.23	0.357	0.77	0.52-1.15	0.204
AA	9 (3.7)	13 (2.7)	1.32	0.55-3.13	0.535	1.26	0.49-3.23	0.634
rs6492763								
TT	89 (37.2)	168 (35.0)	1.00	reference	-	1.00	Reference	-
TC	108 (45.2)	242 (50.4)	0.84	0.60-1.19	0.327	0.80	0.56-1.14	0.220
CC	42 (17.6)	70 (14.6)	1.13	0.71-1.80	0.596	0.99	0.61-1.61	0.971
rs3742106								
AA	86 (35.4)	166 (34.6)	1.00	reference	-	1.00	Reference	-
AC	117 (48.1)	234 (48.8)	0.97	0.68-1.36	0.839	1.00	0.70-1.43	0.992
CC	40 (16.5)	80 (16.7)	0.96	0.61-1.53	0.880	1.06	0.65-1.71	0.823

SNP, single nucleotide polymorphism; OR, *odds ratio*; CI, confidence interval; aOR, adjusted OR for age, gender and smoking habits

CHAPTER V: POLYMORPHISMS IN PROSTAGLANDIN E2 (PGE_2) PATHWAY GENES ALTER THE RISK FOR COLORECTAL ADENOMA RECURRENCE AFTER POLYPECTOMY: A CHANCE FOR INDIVIDUALIZED SURVEILLANCE?

POLYMORPHISMS IN PROSTAGLANDIN E_2 (PGE_2) PATHWAY GENES ALTER THE RISK FOR COLORECTAL ADENOMA RECURRENCE AFTER POLYPECTOMY: A CHANCE FOR INDIVIDUALIZED SURVEILLANCE?

Carina Pereira[1,2,3], Sara Queirós[1], Ana Galaghar[4], Hugo Sousa[1], Ricardo Marcos-Pinto[5], Pedro Pimentel-Nunes[6,7], Catarina Brandão[6], Luís Moreira-Dias[6], Rui Medeiros[1,2,3,8] Mário Dinis-Ribeiro[6,9]

[1]Molecular Oncology Group, Investigation Centre, Portuguese Institute of Oncology, Porto, Portugal; [2]Abel Salazar Institute of Biomedical Sciences, University of Porto, Porto, Portugal; [3]Research Department, Portuguese League Against Cancer, Porto, Portugal; [4]Pathology Department, Portuguese Institute of Oncology, Porto, Portugal; [5]Gastroenterology Department, Centro Hospitalar do Porto, Porto, Portugal; [6]Gastroenterology Department, Portuguese Institute of Oncology, Porto, Portugal; [7]Physiology Department, Faculty of Medicine, University of Porto, Porto, Portugal; [8]CEBIMED, Faculty of Health Sciences of Fernando Pessoa University of Porto, Porto, Portugal; [9]CINTESIS/Department of Biostatistics and Medical Informatics, Faculty of Medicine, University of Porto, Porto, Portugal

Correspondence to:

Carina Pereira

Molecular Oncology Group - IPOP Research Centre

Portuguese Institute of Oncology - Porto,

Rua Dr. Bernardino de Almeida

4200-072 Porto, Portugal.

Tel: +351 22 508 4000 (5115); fax: + 351 22 508 4001

e-mail: anacmpereira@gmail.com

Keywords:

Colorectal adenoma; colorectal adenoma recurrence; Genetic polymorphisms; TagSNPs; Cyclooxygenase-2; 15-Hydroxyprostaglandin Dehydrogenase; Multidrug-Resistance Protein 4; Prostaglandin Transporter

ABSTRACT

Objective: To evaluate the influence of the genetic variability in *COX-2/HPGD/SLCO2A1/ABCC4* prostaglandin E_2 (PGE_2) pathway genes on the development and recurrence of colorectal adenomatous polyps.

Design: A hospital-based case-control study was conducted gathering 480 unscreened individuals and 195 patients with personal history of adenomas. A total of 43 tagSNPs were characterized using the Sequenom platform or real-time PCR.

Results: Ten tagSNPs were identified as susceptibility biomarkers for the development of adenomas: rs689466 in *COX-2*, rs2555639, rs1346271, rs1863642 and rs12500316 in *HPGD*, rs6439448 and rs1131598 in *SLCO2A1* and rs9524821, rs1751051 and rs1678405 in *ABCC4* genes. The haplotype encompassing the rs9524821 and rs1751051 SNPs in *ABCC4* conferred a 3.9 enhanced risk for adenomas onset (95%CI:2.28-6.65, *P*<0.001). Furthermore, the best four-locus gene-gene interaction model included the rs1346271, rs186342 and rs12500316 SNPs in *HPGD* and rs1678405 in *ABCC4* genes and was associated with a 13-fold increased susceptibility (95%CI:3.84-46.3, *P*<0.001, cross-validation (CV) accuracy: 0.78 and CV consistency: 8/10*)*. Interesting, in high-risk patients the rs1678405 *ABCC4* SNP had a lower hazard ratio (HR) and half the crude risk for adenoma recurrence at 36 months, when comparing with the overall high-risk patients (7% vs 14%).

Conclusion: Genetic polymorphisms in the COX-2/PGE_2 pathway appear to contribute to the development of colorectal adenomas and influence the interval time to adenomas recurrence. The improvement of current risk models through the inclusion of genetic biomarkers might provide a better balance between benefits and drawbacks of post-polypectomy surveillance or even target individuals to complementary chemopreventive strategies.

INTRODUCTION

According to the International Agency for Research on Cancer (IACR), 837.437 individuals will be diagnosed and 381.188 will die from CRC in 2020, a 13.6% and 14.4% increase in this cancer burden and mortality from previous estimates [1].

Colorectal adenomatous polyps are well-characterized CRC precursors [2]. Although most adenomas are asymptomatic and do not progress into cancer, the majority of CRC will develop through the adenoma-cancer sequence on an average of 10-15 years [3]. Over one-third of people will develop at least one adenoma in their lifetime [4].

CRC screening has been shown to reduce the incidence and CRC mortality through the endoscopic detection and removal of the precancerous lesions [5,6]. Still, these patients are at increased risk for developing metachronous adenomas or even cancer, with the recurrence rate being around 40-50% [7,8]. Despite population-based CRC screening being widely recommended in Europe, the development of primary prevention strategies is an important goal, considering the inherent limitations of colorectal screening and adherence rates [9,10].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely studied pharmacological agent in CRC prevention and its use reduces the occurrence of advanced adenomas by 28% and the recurrence by 34%, mainly by targeting the cyclooxygenase-2 enzyme (COX-2) [11,12].

Deregulation of COX-2 expression, observed in half of adenomatous polyps, leads to an increased biosynthesis of prostaglandin E_2 (PGE_2) [13]. The pleiotropic effects of higher levels of PGE_2 contribute to key steps of cancer development including stimulation of cell proliferation, angiogenesis, invasiveness and migration, inhibition of apoptosis and immunosurveillance [14]. The degradation of PG is mediated by the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH), encoded by the *hydroxyprostaglandin dehydrogenase* (*HPGD*) gene, which directly counteracts the COX-2 oncogenic PGE_2 pathway [15]. Furthermore, low levels of rectal 15-PGDH were associated with increased adenoma recurrence [16]. The multidrug resistance-associated protein 4 (MRP4) and the prostaglandin transporter (PGT), encoded by the *ATP-binding cassete sub-family C member 4* (*ABCC4*) gene and *solute carrier organic anion transporter family, member 2A1* (*SLCO2A1*) genes, respectively, are the specific prostaglandin membrane

transporters that regulate PGE_2 levels in the extracellular microenvironment [17,18]. PGT and MRP4 mRNA levels were reported to be inversely regulated in human CRC, with PGT expression being repressed and MRP4 up-regulated in CRC tissues and cell lines leading to higher levels of PGE_2 extracellularly thus potentiating the effects of COX-2/PGE_2 pathway [19].

The genetic background certainly contributes to CRC development. At least 35% of CRC cases are attributable to heritability, as reported in a large twin study [20]. Considering that the aforementioned genes are not only highly polymorphic but their expression span several folds, one could hypothesize that an unbalance in PGE_2 levels reflecting potential functional polymorphisms might influence colorectal carcinogenesis and consequently the genetic susceptibility for the development of colorectal precancerous lesions.

Using a tagSNP approach, our group recently reported the involvement of several polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes on CRC development [21]. Therefore, with this study we aimed to investigate whether tagSNPs in these four genes were also associated with earlier stages of colorectal tumor development. To the best of our knowledge this is the first study to evaluate the influence of polymorphisms in *HPGD*, *SLCO2A1* and *ABCC4* genes in the occurrence of colorectal metachronous lesions.

MATERIAL AND METHODS

Type of study

A hospital-based case-control study was design involving a group of unscreened individuals and patients diagnosed with colorectal adenomas. These patients were further stratified according to the presence of metachronous lesions in a retrospective case-cohort study. All individuals were from the northern region of Portugal and recruited at the *Instituto Português de Oncologia do Porto* (IPO-Porto) or *Centro Hospitalar do Porto* (CHP). This project was approved by the Ethics Committee at both institutes (ref. 0084/08 and ref. 080 DEFI/100-CES, respectively) and *Comissão Nacional de Protecção de Dados* (ref. 6619/2011), the Portuguese Data Protection Authority.

Control group

Unscreened individuals between 50 and 75 years of age, without any clinical evidence of CRC or other oncologic malignancy were randomly recruited from the blood donor's service at IPO-Porto between July 2005 and February 2008.

Patients group

Patients diagnosed with one or more adenomas between 1996 and 2008 were enrolled in this study after reviewing a colonoscopy database from the Gastroenterology departments at IPO-Porto and CHP. The inclusion criteria were: (1) age between 50 to 75 years; (2) with a total colonoscopy with good to excellent preparation at diagnosis; (3) without history of inflammatory bowel disease or family history of colorectal tumors; and (4) without previous diagnose of CRC.

Adenoma recurrence was defined as the diagnosis of an adenoma after having a normal total colonoscopy, with a good to excellent preparation, at least one year after the initial diagnosis of adenoma.

Nearly three thousand individuals had history of adenomas, although only less than 10% complied with the inclusion criteria. A total of 256 patients were included in this study. From these we were only able to obtain DNA samples from 195 patients. No differences were observed between demographic variables, lifestyle habits and tumor characteristics between these patients and the overall population of patients.

Sample collection and biological processing

The DNA was extracted from formalin fixed paraffin embedded (FFPE) blocks from the Pathology Departments at both centers, using the GRS Genomic DNA Kit – Tissue, in accordance with the manufacturer's protocol (GRiSP, Porto, Portugal).

Polymorphisms selection

The strategy for polymorphisms selection has been described elsewhere [21]. Briefly, 55 tagSNPs were included after being retrieved from a set of common SNPs in the Caucasian population of HapMap project (CEU) (1) with minor allele frequency equal or superior to 0.15; (2) within the coding region of the genes plus 2Kb upstream and downstream; (3) with a r^2 superior to 0.8 and (4) that successfully converted to the Sequenom platform.

Furthermore, the rs20417, rs689466 and rs5275 polymorphisms in *COX-2* and rs2612656 and rs2555639 in *HPGD* genes, that were previously associated with colorectal tumors development, were also included [22-24].

Genotype characterization

TagSNPs genotyping was performed using MassARRAY iPLEX Gold technology (Sequenom, San Diego, CA) based on multiplexed amplification followed by mass-spectrometric product separation. This technique was carried-out by the *Unidade de Genómica/Serviço de Genotipagem do Instituto Gulbenkian de Ciência.*

The rs689466, rs20417, rs5275, rs2612656 and rs2555639 were characterized through allelic discrimination (Real-Time Polymerase Chain Reaction) using validated TaqMan® SNP genotyping assays (C___2517145_20, C___7550203_10, C___15909858_20, C___16038735_10, respectively) with the exception of the rs20417 SNP which was custom designed (Applied Biosystems, Foster City, California USA).

Quality control

Genotypes were excluded from the analysis if any of the following criteria was applied: call rate inferior to 0.90; concordance rate inferior to 0.95 and Hardy-Weinberg equilibrium (HWE) with $P<0.05$. Blank templates were included in each 96 and 384-well plates to ensure contamination-free results. Two researchers performed the genotype interpretation independently and five to ten percent of all samples were randomly selected and re-submitted to a new genetic characterization. Furthermore, the use of FFPE samples for SNP genotyping was previously validated by comparing the genotypes from 20 DNAs isolated from fresh peripheral blood and paired FFPE samples from CRC patients.

Statistical analysis

The Hardy–Weinberg equilibrium was tested by the Pearson's goodness-of-fit test to compare the observed versus the expected genotype distribution among the control population.

Data analysis was performed using the computer software IBM Statistical Package for Social Sciences-SPSS (IBM Corp., Armonk, New York, USA) for Macintosh (version 19.0). Chi-square analysis was used to compare categorical variables,

using a 5% level of significance. Non-parametric tests were used to compare mean values. Odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measure of the association between the genetic variants and the risk for the development of CRC. Covariates expected to be involved on colorectal carcinogenesis, namely age, gender and smoking habits were included in the logistic regression analysis. A bootstrap resampling was used to assess the stability of risk estimates (1000 replications). The false positive report probability (FPRP) was used to confirm the noteworthiness of significant findings on the overall risk for colorectal adenoma development, according to the study by Wacholder and colleagues [25]. The FPRP threshold was set at 0.5 under an assigned prior probability ranging from 0.01 to 0.10 to detect an OR of 1.5.

Haplotype analysis was performed at a gene level using the SNPStats software (www. http://bioinfo.iconcologia.net/SNPstats). The haplotype frequencies were estimated using the implementation of the EM algorithm coded into the *haplo.stats* package. The most frequent haplotype was automatically selected as the reference category. After excluding the genetic variations most likely to represent false positive findings, all polymorphisms with significant associations were included within each gene.

The open-source multifactor dimensionality reduction (MDR) software (version 3.0.2) (www.epistasis.org) was used to assess potential gene-gene interactions between SNPs with statistical significant impact on colorectal adenoma genetic susceptibility. The fitness of an MDR model was estimated by determining the testing accuracy and its cross-validation consistency (CVC). Using a 10-fold cross-validation method the data was divided into 10 sets, in which 9 subsets were training sets and one subset was a test set. Hence, the CVC is a measure of the number of times of 10 divisions of the dataset the best model was extracted.

Kaplan-Meier curves were used to evaluate the correlation between the genetic variants and time to recurrence; log rank statistical test was used for curves comparison.

RESULTS

Study population

A description of the population understudied is displayed in Table 1 and as can be observed patients with history of adenomas were slightly older than controls' (61 vs 58, *P*<0.001). Males and non-smokers were overrepresented in either group (57 vs 65%, *P*=0159 and 72 vs 70%, *P*=0.075, respectively).

In over 70% of patients, less than three adenomas (71%) were detected. Most were located distally to the splenic flexure (82%) and were larger or equal to 10 mm in size (64%). Histologically, high-grade dysplasia was described in 33% of index adenomatous polyps.

High-risk patients for adenoma recurrence (adenoma with villous histology or high grade dysplasia or ≥ 10 mm in size, or ≥ 3 adenomas) represented 72% of cases' population. The median follow up time was 76 months (22-201) and metachronous lesions were identified in 46% of patients with personal history of adenomas. No differences were observed between these patients and the ones without adenoma recurrence during the follow up period.

Table 1. Description of population

	Controls (n=480)	Adenomas (n=195)	*P* value	Recurrence		*P* value
				No	Yes	
Demographics						
Age (years)						
Mean (SD)	58 (4.90)	61 (6.78)		61 (6.64)	61 (6.74)	
Median (min-max)	58 (50-69)	61 (50-75)	<0.001	61 (50-75)	60 (50-75)	0.698
Gender, *n* (%)						
Male	314 (65.4)	110 (57.3)		54 (49.5)	55 (50.5)	
Female	166 (34.6)	82 (42.7)	0.159	48 (60.0)	32 (40.0)	0.154
Lifestyle behaviors						
Smoking status, *n* (%)						
Never-smokers	219 (60.3)	86 (71.7)		47 (54.7)	39 (45.3)	
Ever-smokers[£]	144 (39.7)	34 (28.3)	0.075	16 (47.1)	18 (52.9)	0.453
High-risk patients*						
No	-	53 (27.9)		27 (54.0)	23 (46.0)	
Yes	-	137 (72.1)		74 (53.6)	64 (46.4)	0.963
Time of follow up (mo)						
Mean (SD)	-	80.1 (39.5)		-	-	-
Median (min-max)	-	76 (22-201)		-	-	-
Polyps characteristics[#]						
Number of adenomas						
Mean (SD)	-	2.15 (1.69)		1.98 (1.51)	2.38 (1.87)	
Median (min-max)	-	1 (1-10)		1 (1-9)	2 (1-10)	0.119
<3	-	133 (70.7)		74 (55.6)	59 (44.4)	
≥3	-	55 (29.3)		27 (49.1)	28 (50.9)	0.413
Tumor location, *n* (%)						
Distal	-	155 (82.0)		87 (56.1)	68 (43.9)	
Proximal	-	34 (18.0)		15 (44.1)	19 (55.9)	0.203
Size, *n* (%)						
<10	-	67 (35.6)		37 (55.2)	30 (44.8)	
≥10	-	121 (64.4)		65 (53.7)	56 (46.3)	0.843
Morphology, *n* (%)						
Pedunculated	-	76 (46.1)		42 (55.3)	34 (44.7)	
Sessile	-	89 (53.9)		42 (47.2)	47 (52.8)	0.301
Histological Grade, *n* (%)						
Low-grade dysplasia	-	127 (67.2)		67 (52.8)	60 (47.2)	
High-grade dysplasia	-	62 (32.8)		35 (56.5)	27 (43.5)	0.632
Histological type, n (%)						
Tubular	-	30 (46.1)		16 (53.3)	14 (46.7)	
Tubulovillous	-	20 (30.8)		14 (70.0)	6 (30.0)	
Villous	-	15 (23.1)		9 (60.0)	6 (40.0)	0.446
Metachronous adenomas						
No	-	102 (54)		-	-	
Yes	-	87 (46)		-	-	

SD, standart deviation; min, minimum; max, maximum; mo, months
[£]Former and current smokers pooled together
*risk stratification for adenoma recurrence based on the endoscopic findings at baseline colonoscopy. Low-risk patients: 1-2 tubular adenomas <10 mm in size with low grade dysplasia; High-risk patients: patients with adenomas with villous histology or high grade dysplasia or ≥ 10 mm in size, or ≥ 3 adenomas;
[#]the most advanced tumor was considered

Risk estimates for colorectal adenoma

Eight SNPs were excluded from the analysis due to genotyping failure and four SNPs were dropped because their frequencies deviated from HWE (P<0.05). A total of 43 SNPs were included in the risk estimate analysis. The mean genotype call and concordance rates were 0.97. The description of selected SNPs and genotype distribution are displayed in Table 1 and Table 2 of supporting information, respectively.

Using the false positive report probability (FPRP), nine polymorphisms retained their association with colorectal adenoma development, as observed in Table 2. Individuals homozygous for the minor G allele of the rs689466 SNP in *COX-2* gene were overrepresented in the group of cases (10% vs 3%, in controls) leading to a 3-fold increased risk for colorectal adenoma (95%CI:1.52-6.86, P=0.002) in the multivariate analysis, including age, gender and smoking habits as covariates. Following a recessive model, the rs2555639 polymorphism was positively linked with the onset of colorectal precancerous lesions (OR=2.48; 95%CI:1.36-4.53, P=0.003), whereas the rs1346271, rs1863642 and rs12500316 genetic variants in the *HPGD* gene were associated with a 45-51% protection in carriers of the minor alleles (OR=0.55; 95%CI:0.35-0.85, P=0.008, OR=0.55; 95%CI:0.36-0.85, P=0.007 and OR=0.49; 95%CI:0.31-0.78, P=0.002 for the rs1346271, rs1863642 and rs12500316, respectively). Stressing the *SLCO2A1* gene, individuals carrying the rs6439448G allele presented a 62% decreased risk for colorectal adenomas (95%CI:0.22-0.65, P<0.001). Although with a lower influence, the rs1131598 was also inversely associated with colorectal adenomas development (OR=0.58; 95%CI:0.41-0.84, P=0.018). Three out of the 21 polymorphisms in *ABCC4* gene appeared to have an impact in colorectal carcinogenesis in early stages. The AA and CC genotypes of rs9524821 and rs1751051 polymorphisms, more than double the susceptibility for colonic precancerous tumors (OR=2.38; 95%CI:1.39-4.09, P=0.002 and OR=2.75; 95%CI:1.58-4.80, P<0.001). In contrast, a protective role was noticed for genotypes carrying the rs1678405C allele SNP was noticed, even more in homozygous for the C allele (OR=0.41; 95%CI:0.27-0.63, P<0.001).

Table 2. Risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* genes in colorectal adenoma development

SNP	Model of inheritance	aOR	95%CI	*P* value	$P_{bootstrap}$	FPRP prior probability		
						0.01	0.05	0.1
COX-2								
rs689466	Recessive (AA/AGvsGG)	3.23	1.52-6.86	0.002	0.001	0.908	0.654	**0.472***
HPGD								
rs2555639	Recessive (TT/TCvsCC)	2.48	1.36-4.53	0.003	0.002	0.859	0.538	**0.356***
rs2612656	Dominant (AAvsAG/GG)	0.47	0.23-0.94	0.033	0.035	0.953	0.794	0.646
	Recessive (AA/AGvsGG)	3.20	1.22-8.41	0.018	0.009	0.967	0.848	0.726
rs8752	Recessive (AA/AGvsGG)	1.94	1.09-3.44	0.023	0.036	0.924	0.701	0.526
rs1346271	Dominant (GGvsGC/CC)	0.55	0.35-0.85	0.008	0.013	0.785	**0.411**	**0.249**
rs1863642	Dominant (GGvsGT/TT)	0.55	0.36-0.85	0.007	0.008	0.785	**0.411**	**0.249**
rs12500316	Dominant (CCvsCT/TT)	0.49	0.31-0.78	0.002	0.002	0.729	**0.340**	**0.196**
SLCO2A1								
rs4241362	Recessive (TT/TCvsCC)	3.90	1.80-8.43	0.001	0.001	0.876	0.575	**0.391**
rs6439448	Dominant (CCvsCA/AA)	0.38	0.22-0.65	<0.001	0.002	0.670	**0.280**	**0.156**
rs9821091	Dominant (GGvsGA/AA)	0.62	0.40-0.96	0.033	0.044	0.895	0.621	**0.437**
	Recessive (GG/GAvsAA)	1.77	1.02-3.06	0.041	0.048	0.936	0.738	0.571
rs4241365	Recessive (TT/TCvsCC)	2.60	1.30-5.22	0.007	0.006	0.921	0.692	0.516
rs7625035	Recessive (AA/AGvsGG)	2.68	1.71-6.11	0.020	0.026	0.957	0.812	0.672
rs1131598	Dominant (AAvsAG/GG)	0.58	0.41-0.84	0.018	0.052	0.629	**0.245**	**0.133**
rs10935090	Recessive (CC/CTvsTT)	5.18	1.33-20.17	0.018	0.002	0.979	0.901	0.812
ABCC4								
rs9524821	Recessive (GG/GAvsAA)	2.38	1.39-4.09	0.002	0.003	0.780	**0.405**	**0.244**
rs869951	Dominant (GGvsGC/CC)	0.60	0.39-0.92	0.018	0.019	0.858	0.537	**0.354**
rs1751051	Recessive (TT/TAvsAA)	2.75	1.58-4.80	<0.001	0.001	0.691	**0.300**	**0.169**
rs2892713	Recessive (CC/CTvsTT)	2.50	1.12-5.58	0.025	0.006	0.959	0.819	0.682
rs4612933	Recessive (CC/CTvsTT)	3.03	1.35-6.79	0.007	0.005	0.941	0.754	**0.593**
rs4148476	Recessive (TT/TGvsGG)	3.22	1.41-7.36	0.005	0.005	0.940	0.751	0.588
rs1678405	Dominant (TTvsTC/CC)	0.41	0.27-0.63	<0.001	0.001	**0.261**	**0.064**	**0.031**
	Recessive (TT/TCvsCC)	0.15	0.04-0.63	0.010	0.010	0.979	0.897	0.805
rs1751031	Recessive (AA/AGvsGG)	2.99	1.11-8.00	0.030	0.016	0.971	0.867	0.756
rs7993878	Recessive (GG/GAvsAA)	3.14	1.09-9.01	0.033	0.024	0.975	0.882	0.780

SNP, single nucleotide polymorphism; FPRP, false positive report probability; Bold for FPRP<0.5; *A prior probability of 0.1 was assumed for the rs689466 and rs2555369 considering the available epidemiologic and functional data; For all other polymorphisms a prior probability of 0.05 was considered.

aOdds Ratio, Logistic regression (Forward:conditional model) including gender, smoking habits and age (median global age of 59 years used as cutoff). CI, confidence interval; Only statistical significant associations are presented (*P*<0.05); bootstrap results are based in 1000 samples.

Haplotype analysis for colorectal adenomas

Since multiple SNPs were addressed within each gene and multiple associations were reported, with exception of *COX-2* gene, a haplotype analysis was performed. The frequencies of derived haplotypes from *HPGD*, *SLCO2A1* and *ABCC4* genes are presented in Table 3. The most frequent haplotype of the *HPGD* gene, the CGGT, was present in 35% of controls and used as the reference one. The blocks containing the rs1250016T allele, TGGT, and the rs1346271C-rs1863642T-rs2555639C alleles, CCTC, were associated with a high protection, although they were detected roughly in 1% of cases (OR=0.05; 95%CI:0.01-0.15, *P*<0.001 and OR=0.06; 95%CI:0.01-0.33, *P*=0.001, respectively). Consistent with the individual SNP analyses, the haplotypes carrying the rs1131598G allele, GC, or rs6439448A allele, AA, displayed a protective role for the development of colorectal adenoma (vs AC, OR=0.68; 95%CI:0.49-0.95, *P*=0.024 and OR=0.50; 95%CI:0.33-0.76, *P*=0.001, respectively). No block containing both the minor alleles was present at a frequency higher or equal to 5%. Out of the six haplotypes described for the *ABCC4* gene, the block carrying the rs9524821A and rs1751051A alleles boosted even further the susceptibility for colorectal precancerous lesion reported in the individual analysis in contrast with the GTT haplotype (OR=3.90; 95%CI: 2.28-6.65, *P*<0.001).

Gene-gene interaction analysis in colorectal adenoma

To address possible interactions between the noteworthy SNPs from the main analysis, an exhaustive MDR approach was employed and Table 4 summarizes the best interactive models obtained. All best models from one to four-locus were significant at *P*≤0.001, and the highest cross-validation consistency was observed with the two-factor interaction model (9/10). Nevertheless, the best four-locus model achieved the highest testing accuracy of 78% for predicting the development of colorectal adenomas, although with a lower cross-validation consistency (8/10). This interaction model included the rs1346271, rs1863642 and rs12500316 polymorphisms in *HPGD* gene and rs1678405 in *ABCC4* gene and was associated with a 13-fold increased adenoma risk (95%CI:3.84-46.3, *P*<0.001).

Table 3. Haplotype frequencies between patients and controls and risk estimates for their involvement in adenoma development

Gene / Haplotype	% Cases	% Controls	aOR	95%CI	*P* value
*HPGD**					
C-G-G-T	35.0	28.0	1	Reference	-
C-G-G-C	18.6	10.9	1.04	0.64-1.70	0.87
C-C-G-T	15.3	9.1	1.11	0.68-1.82	0.67
T-G-G-T	1.1	12.6	0.05	0.01-0.15	**<0.001**
C-C-T-T	0	10.4	-	-	-
C-C-T-C	0.8	8.8	0.06	0.01-0.33	**0.001**
SLCO2A1[£]					
A-C	71.6	60.8	1	Reference	-
G-C	17.2	20.8	0.68	0.49-0.95	**0.024**
A-A	8.4	15.4	0.50	0.33-0.76	**0.001**
ABCC4[¥]					
G-T-T	19.8	26.9	1	Reference	-
A-T-T	26.8	19.7	1.85	1.19-2.86	**0.006**
G-C-T	15.3	17.0	1.18	0.71-1.98	0.52
G-T-A	14.3	14.4	1.51	0.89-2.56	0.13
A-T-A	17.3	3.9	3.90	2.28-6.65	**<0.001**
G-C-A	5.7	6.1	0.99	0.46-2.12	0.98

Bold for *P*<0.001
aOdds ratio (OR) adjusted for age (categorical variable, using the global median age of 59 years as cutoff), gender and smoking habits: CI, confidence interval
*SNPs order: rs12500316-rs1346271-rsrs1863642-rs2555639
[£]SNPs order: rs1131598-rs6439448
[¥]SNPs order: rs9524821-rs1678405-rs1751051

Table 4. MDR analysis for the colorectal adenoma risk prediction

Best model	CV accuracy	CV consistency	aOR	95%CI	*P* value
rs1346271, rs12500316	0.6964	9/10	5.41	1.88-15.5	**0.001**
rs1346271, rs1863642, rs12500316	0.7006	6/10	5.51	1.90-15.9	**0.001**
rs1346271, rs1863642, rs12500316, rs1678405	0.7816	8/10	13.3	3.84-46.3	**<0.001**

Bold for *P*<0.001
MDR, Multifactor dimensionality reduction; CV, cross-validation; aOR, Odds ratio adjusted for age, gender and smoking habits; CI, confidence interval

Risk assessment for the development of colorectal metachronous lesions

The genetic variability in *COX-2* and *HPGD* genes does not appear to contribute to the development of metachronous tumors in patients previously diagnosed with colorectal adenomas, as observed in Table 5.

Two polymorphisms in *SLCO2A1* gene were reported to influence the susceptibility for colorectal adenomas recurrence. The rs1131598GG homozygous genotype was associated with an enhanced risk of 6.3 (95%CI:1.31-30.0, *P*=0.021). On the other hand, individuals carrying the rs7340717T allele had a 56% protection for developing metachronous adenomas (95%CI:0.20-0.97, *P*=0.041). Under a dominant model of inheritance, the rs1751031 and rs9524821 polymorphisms in *ABCC4* gene appear to display a protective behavior (OR=0.29; 95%CI:0.12-0.72, *P*=0.007 and OR=0.42; 95%CI:0.19-0.93, *P*=0.033, respectively), in contrast with the positive association observed in the presence of rs8002180C allele (OR=2.22; 95%CI:1.04-4.76, *P*=0.041). None of the aforementioned SNPs retained their noteworthiness upon the FPRP analysis, potentially indicating false positive findings (FPRP>0.5).

Table 5. Risk estimates for the influence of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal adenoma recurrence

SNP	Model of inheritance	aOR	95%CI	*P* value	$P_{bootstrap}$	FPRP prior probability		
						0.01	0.05	0.1
SLCO2A1								
rs1131598	Recessive (AA/AGvsGG)	6.28	1.31-30.0	0.021	0.019	0.983	0.918	0.841
rs7340717	Dominant (GGvsGT/TT)	0.44	0.20-0.97	0.041	0.060	0.965	0.840	0.713
ABCC4								
rs1751031	Dominant (AAvsAG/GG)	0.29	0.12-0.72	0.007	0.015	0.954	0.799	0.653
rs8002180	Dominant (TTvsTC/CC)	2.22	1.04-4.76	0.041	0.057	0.962	0.831	0.700
rs9524821	Dominant (GGvsGA/AA)	0.42	0.19-0.93	0.033	0.034	0.961	0.827	0.694

SNP, single nucleotide polymorphism; FPRP, false positive report probability
aOdds Ratio: Logistic regression (Forward: conditional method) including smoking habits, risk profile and centre as covariates.; CI, confidence interval; bootstrap results are based in 1000 samples
Only statistcal significant association are presented (*P*<0.05)

Influence on the time to recurrence and crude risk

We next, inquired if polymorphisms in these key genes in PGE_2 pathway could influence not only the time but also the crude risk for adenomas recurrence at 36, 60 and 120 months, following the recommendations for post-polypectomy colonoscopy surveillance (Table 6) [26]. Although no difference was observed on the time to adenoma recurrence (112 vs 105 months, *P*=0.788) or recurrence rate (46%, *P*=0.996) between the high and low-risk patients, 14% of all adenomatous polyps recurred at 36 in the high-risk group in contrast to the 2% reported in low-risk patients. Additionally, nearly 95% (18/19) of metachronous advanced adenomas were described in the high-risk group, with 28% and 67% being diagnosed at 36 and 60 months (data not shown).

The contribution of the genetic background appeared to be particularly relevant on patients at low-risk. For example, the rs9524821AA genotype not only was associated with a nearly three-fold increased susceptibility in the cox regression analysis (95%CI:1.07-8.03, *P*=0.036), but also half of patients carrying this genotype had adenoma recurrence at 60 months, considerable higher than the 21% noticed in low-risk patients. Similarly, patients' carriers of rs2274403AA genotype had a lower interval until recurrence (85 (29-140) vs 122 (109-135), *P*=0.011) with 44% of metachronous tumors developing by 36 months (vs 23% for AG/GG). In the high-risk group the genetic polymorphism with potential for influencing current guidelines is the rs1678405, for which TT carriers had a lower hazard ratio, with a higher time to recurrence (109 (89-129) vs 90 (76-104), *P*=0.075) and half the crude risk for recurrence at 36 months, in comparison with the overall high-risk patients (7% vs 14%).

Table 6: Influence of genetic variations in *COX-2/HPGD/SLCO2A1/ABCC4* on the time to recurrence of colorectal adenomas and crude risk of recurrence at 36, 60 and 120 months of follow up.

		Recurrence, %	aOR (95%CI)	aHR (95%CI)	Time to recurrence* (min-max)	Crude risk for recurrence, ‰		
						36 mo	60 mo	120 mo
Low-risk individuals								
Global		46			112 (100-124)	2	21	86
SLCO2A1								
rs9820625	AA/AC	32	10.71	3.33	115 (100-130)	3	16	70
	CC	80	(1.17-98.24)	(1.22-9.10)	85 (62-108)	0	29	100
rs9524821	GG/GA	41	-	2.93	122 (97-147)	4	16	78
	AA	43		(1.07-8.03)*	107 (57-157)	0	48	100
ABCC4								
rs1678396	TT	50	-	0.20	94 (90-98)	0	18	100
	TC/CC	39		(0.07-0.60)	122 (95-149)	3	18	80
rs2274403	AA	50	-	0.26	85 (29-140)	0	44	69
	AG/GG	39		(0.08-0.83)	122 (109-135)	3	23	83
rs3742106	AA	21	5.36	5.78	135 (77-193)	0	8	72
	AC/CC	59	(1.25-23.04)	(1.61-20.8)	105 (84-126)	3	27	92
rs6492763	TT	73	0.18	0.26	93 (55-130)	6	38	100
	TC/CC	15	(0.04-0.74)	(0.07-0.91)	176 (-)	0	7	66
rs869951	GG/GC	35	-	-	114 (105-123)	3	17	89
	CC	75			66 (48-84)	0	50	100
High-risk individuals								
Global		46			105 (87-123)	14	27	82
SLCO2A1								
rs1131598	AA/AG	44	-	3.23	105 (86-124)	13	23	81
	GG	73		(1.49-7.02)	67 (59-74)	19	59	100
rs7616492	GG	57	-	-	94 (78-110)	14	40	94
	GA/AA	39			121 (98-143)	13	20	74
rs7340717	GG/GT	44	-	-	115 (88-142)	12	26	81
	TT	57			94 (43-145)	26	47	93
ABCC4								
rs1678405	TT	39	2.09*	1.75*	109 (89-129)	7	22	77
	TC/CC	57	(1.04-4.23)	(1.05-2.91)	90 (76-104)	23	35	88

aOdds Ratio: Logistic regression (Forward: conditional method) including centre as covariate; aHazard Ratio: Cox regression (Forward: conditional method) including centre as covariate; CI, confidence interval; min, minimum; max, maximum; mo, month.
*P>0.05 upon the bootstrap analysis (bootstrap results are based in 1000 samples)

DISCUSSION

Colorectal cancer still remains a major clinical and public health challenge that could be averted by applying the current knowledge about CRC prevention and improving the adherence to established screening guidelines [5,6].

The rapid decline on CRC incidence over the past decade has been largely attributed to the endoscopic detection and removal of precancerous adenomatous polyps and endoscopic follow up of these patients with personal history of colorectal adenomas [5]. Nevertheless, not only are the compliance rates far from

the desirable, even in countries with implemented population-based CRC screening guidelines, also important lesions are missed or incompletely removed during colonoscopy [27,28].

The search for susceptibility biomarkers in colorectal carcinogenesis might reveal an important tool to select unscreened individuals to CRC screening or even to complementary chemopreventive strategies with NSAIDs by allowing the identification of individuals at higher risk for the development of colorectal tumors. Currently, NSAIDs use in CRC prevention is hampered by the adverse gastrointestinal side effects associated with its regular use in average risk populations [29].

The efflux-dominated flow of PG during carcinogenesis as a reflection of an increased expression of COX-2 and MRP4 and down regulation of 15-PGDH and PGT leads to an accumulation of PGE_2 in the extracellular milieu culminating in the activation of a plethora of pathways that stimulate tumor development [19].

In the present study, we addressed the role of 43 tagSNPS in four candidate genes (*COX-2/HPGD/SLCO2A1/ABCC4*) of COX-2/PGE_2 pathway on the development and recurrence of colorectal adenomatous polyps in a Northern Portuguese population. Recently, using the same tagSNPs approach and targeting the same pathway we also identified the rs689466A>G polymorphism in *COX-2*, the rs1346271G>C in *HPGD*, the rs6439448C>A in *SLCO2A1* and the rs1751051T>A in *ABCC4* genes polymorphisms as susceptibility biomarkers for CRC, supporting the associations reported here and the role they might portray in colorectal carcinogenesis [21].

The homozygous GG genotype for the rs689466 SNP, also known as -1195A>G *COX-2* polymorphism, associated presently with a three-fold higher predisposition, was previously related with a higher risk for duodenal adenomatosis in patients with familial adenomatous polyposis (FAP) [30]. Although representing a hereditary syndrome, deregulation of COX-2 expression was observed in normal and duodenal adenomas of FAP patients.[31] Furthermore, our group in a earlier study, observed a higher transcriptional activity in HCT116 and HCA-7 CRC cell lines transfected with COX-2 promoters' encompassing the rs689466G allele, thus providing a biological plausibility for the epidemiologic observations [32].

Thompson and colleagues [24], first associated the rs2555639T>C SNP located at 17.74kb upstream the 5'UTR of *HPGD* gene with a 40% increased risk for CRC in

TT homozygous carriers. Surprisingly, in our population this SNP not only appears to be more relevant in early stages of colorectal carcinogenesis, but the opposing rs2555639CC genotype was the one linked to colorectal adenomas onset. This conflicting data, might reflect population stratification involving different genetic ancestry, considering that the initial study involved participants from the Kentucky Surveillance, Epidemiology and End Results (SEER) registry most likely with Northern or Western European ancestry (English, German, Irish ancestry).

Furthermore, the rs1346271G>C, rs1863642G>T and rs12500316C>T tagSNPs in *HPGD* gene displayed a protective role in colorectal adenoma onset, as reported by Edwards and colleagues [33]. Apart from the rs1346271 polymorphism, that was previously associated with a reduced risk for CRC and locates in *HPGD* promoter region altering the binding site for nuclear proteins (SNPinfo software), no other SNP provides a biological reasoning for the protection observed [21].

The PGT and MRP4 specific PG membrane transporters are encoded by highly polymorphic genes. Still, the study of genetic variants in *SLCO2A1* and *ABCC4* genes on the etiology of malignant diseases has been rather neglected [21,34].

In our population, two polymorphisms in *SLCO2A1* gene appeared to modulate the susceptibility for colorectal adenoma (rs1131598A>G and rs6439448C>A), although it was more noticeable in rs6439448A allele carriers, in whom a 60% protection was observed. Biologically, the rs6439448 SNP tags two other polymorphisms with predicted impact on PGT expression: the rs2370512T>A located in the 3'UTR could affect the binding of microRNAs and stability of mRNA and the nonsynonymous rs34550074G>A SNP at codon 396 codes for two different amino acids (Ala396Thr) with potential repercussion on protein structure and function. Similarly, the rs1131598A>G polymorphism, located at 3'UTR, is predicted to influence mRNA stability and thus the PGT protein expression.

Remarkably, homozygous mutations in *HPGD* and more recently in *SLCO2A1* gene were identified as causative agents for the development of primary hypertrophic osteoarthropathy (PHO) [35,36]. Similarly to neoplastic tumor genesis, increased levels of PGE_2 play a role in the pathogenesis of PHO, thus reinforcing the impact that genetic variability in these genes might portray in disease development by disrupting the normal 15-PGDH and PGT levels or activity [36].

Regarding the *ABCC4* gene, three polymorphisms influenced the risk for adenoma onset (rs1678405, rs9524821 and rs1751051). More interesting, individuals

carrying the haplotype containing both the A alleles for the rs9524821 and rs1751051 SNPs had a nearly 4-fold increased susceptibility. The *in silico* analysis did not provide any biological clue for the involvement of these polymorphisms in MRP4 expression or function.

The common disease-common variant (CD-CV) hypothesis predicts that complex polygenic diseases develop from the additive or multiplicative effect of low penetrance genes [37]. Here, a 13-fold increased predisposition was noticed in the multi-locus analysis, supporting the role that common variants portray in colorectal carcinogenesis.

The current post-polypectomy guidelines recommend endoscopic surveillance based on risk stratification upon the endoscopic findings at baseline colonoscopy [26]. In this study we observed that polymorphisms in the COX-2/PGE2 pathway, particularly on the *ABCC4* gene, influenced not only the hazard ratio for the development of metachronous adenomas, but perhaps more importantly the probability for recurrence considering the surveillance intervals currently recommended. As an example, the individuals carrying the rs9524821AA genotype in the low-risk group presented a nearly three-fold increased hazard ratio for adenoma recurrence and nearly half of them developed metachronous lesions by 60 months (vs 16%, for G allele carriers). In contrast, the rs1678405TT genotype was associated with a 7% recurrence at 36 months in the high-risk group, in opposition to C allele carriers (23%). Considering the limited number of participants in this study we were not able to specifically assess the influence of these SNPs on the risk for the development of advanced colorectal adenomas, but assuming an equal distribution among genotypes, this could represent that patients carrying the rs1678405TT genotype might benefit from a looser surveillance interval.

These observations should be interpreted with caution considering the several drawbacks encountered in this study. Inherent to a retrospective study we cannot rule out selection bias, even more considering that our control population was represented by unscreened individuals. Nevertheless, if this were true we would expect stronger associations; or recall bias that could decrease the availability and accuracy of collected data, compromising our ability to estimate possible gene-environment interactions. Our major limitation certainly falls on the low statistical

power (<80%), namely when carrying out the analysis on the recurrence of adenomas. Although, the bootstrap analysis reinforced the robustness of the associations reported here, further replication studies with larger and independent populations are needed to clarify the involvement of these polymorphisms in early stages of colorectal carcinogenesis. Furthermore, functional studies evaluating the repercussion of the aforementioned SNPs on protein expression/function will allow a deeper understanding of their real contribution on cancer development.

In this study, we observed the involvement of several polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes in colorectal adenoma development and recurrence. Furthermore, the incorporation of genetic variants in current risk models might provide a better balance between benefits and drawbacks of post-polypectomy surveillance.

REFERENCES

[1] Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer 2013. Available from: http://globocan.iarc.fr, accessed on 17/04/2014.

[2] Levine JS, Ahnen DJ. Clinical practice. Adenomatous polyps of the colon. N Engl J Med 2006;355(24):2551-7.

[3] Kelloff GJ, Schilsky RL, Alberts DS, et al. Colorectal adenomas: a prototype for use of surrogate end points in the development of cancer prevention. Clin Cancer Res 2004;10(11):3908:18.

[4] Lieberman DA, Weiss DG, Bond JH, et al. Use of colonoscopy to screen asymptomatic adults for colorectal cancer. N Engl J Med 2000;343(3):162-8.

[5] Atkin WS, Edwards R, Kralj-Hans, et al. Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a muiticentre randomized controlled trial. Lancet 2010;375:1624-33.

[6] Zauber AG, Winawer SJ, O'Brien MJ, et al. Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. N Engl J Med 2012;366:687-96.

[7] Yamaji Y, Mitsushima T, Ikuma H, et al. Incidence and recurrence rates of colorectal adenomas estimated by annually repeated colonoscopies on asymptomatic Japanese. Gut 2004;53:568–72.

[8] Cottet V, Jooste V, Fournel I, et al. Long-term risk of colorectal cancer after adenoma removal: a population-based cohort study. Gut. 2012;61(8):1180-6.

[9] Levin TR, Zhao W, Conell C, et al. Complications of colonoscopy in an integrated heath care delivery system. Ann Intern Med 2006;145(12):880-6.

[10] Pox CP, Altenhofen L, Brenner H, et al. Efficacy of a nationwide screening colonoscopy program for colorectal cancer. Gastroenterology 2012;142(7):1460-7.

[11] Cole BF, Logan RF, Habali S, et al. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. J Natl Cancer Inst 2009;101(4):256-66.

[12] Gao F, Liao C, Liu L, et al. The effect of aspirin in the recurrence of Colorectal adenomas: a meta-analysis of randomized controlled trials. Colorectal Dis 2009;11(9):893-901.

[13] Eberhart CE, Coffey RJ, Radhika A, et al. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107:1183-8.

[14] Wang D, Mann JR, Dubois RN. The role of prostaglandin and other eicosanoids in gastrointestinal tract. Gastroenterology 2005;128:1445-61.

[15] Tai HH, Ensor CM, Tong M, et al. Prostaglandin catabolizing enzymes. Prostaglandins Other Lipid Mediat 2002;68-69:483-93.

[16] Yan M, Myung SJ, Fink SP, et al. 15-Hydroxyprostaglandin dehydrogenase inactivation as a mechanism of resistance to Celecoxib chemoprevention of colon tumors. Proc Natl Acad Sci 2009;106(23):9409-13.

[17] Reid G, Weilinga P, Zelcer N, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflamatory drugs. Proc Natl Acad Sci 2003;100(16):9244-9.

[18] Schuster VI. Prostaglandin transport. Prostaglandins Other Lipid Mediat 2002;68-69:633-47.

[19] Holla VR, Backlund MG, Yang P, et al. Regulation of prostaglandin transporters on colorectal neoplasia. Cancer Prev Res 2008;1(2):93-9.

[20] Lichtenstein P, Holm NV, Verkasalo PK, et al. Environmental and heritable factors in the causation of cancer-analyses of cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med 2000;343(2):78-85.

[21] Pereira C, Queirós S, Galaghar A, et al. Genetic variability in key genes in prostglandin E2 pathway (COX-2/HPGD/ABCC4/SLCO2A1) and their involvement in colorectal cancer develpment. PLoS One. 2014 Apr 2;9(4):e92000. doi: 10.1371/journal.pone.0092000. eCollection 2014.

[22] Pereira C, Medeiros R, Dinis-Ribeiro M. Cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis: are conclusive results available? Eur J Gastroenterol Hepatol 2009;21:76-91.

[23] Hoeft B, Linseisen J, Beckmann L, et al. Polymorphisms in fatty acid metabolism-related genes are associated with colorectal cancer risk. Carcinogenesis 2010;31(3):466-72.

[24] Thompson CL, Fink SP, Lutterbaugh JD, et al. Genetic variation in 15-Hydroxyprostaglandin dehydrogenase and colon cancer susceptibility. PLoS One 2013;8(5):e64122.

[25] Wacholder S, Chanock S, Garcia-Closas, et al. Assessing the probability that a positive repor is false: an approach for molecular epidemiology studies. J Natl Cancer Int 2004;96:434-42.

[26] Hassan C, Quintero E, Dumonceau J-M, et al. Post-polypectomy colonoscopy surveillance: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. Endoscopy 2013;45:842-51.

[27] van Rijn JC, Reitsma JB, Stoker J, et al. Polyp miss rate determined by tandem colonoscopy: a systematic review. Am J Gastroenterol 2006;101:343-50.

[28] Robertson DJ, Greenberg ER, Beach M, et al. Colorectal cancer in patients under close colonoscopic surveillance. Gastroenterology 2005;129:34-41.

[29] Rodriguez LA, Tolosa LB. Risk of upper gastrointestinal complications among users of Traditional NSAIDs and COXIBs in te general population. Gastroenterology 2009;132(2):4498-506.

[30] Peters WH, te Morsche RH, Roelofs HM, et al. COX-2 polymorphisms in patients with familial adenomatous polyposis. Oncol Res 2009;17(8):347-51.

[31] Brosens LA, Jacubuzio-Donahue CA, Keller JJ, et al. Increased cyclooxygenase-2 expression in duodenal compared with colonic tissues in familial adenomatous polyposis and relationship to the -765G -> C COX-2 polymorphism. Clin Cancer Res 2005;11(11):4090-6.

[32] Pereira C, Sousa H, Silva J, et al. The -1195G allele increases the transcriptional activity of cyclooxygenase-2 gene (COX-2) in colon cancer cell lines. Mol Carcinog 2014;53 Suppl 1:E92-5.

[33] Edwards TL, Shrubsole MJ, Cai Q, et al. A study of prostaglandina pathway genes and interactions with current nonsteroidal anti-inflammatory drug use in Colorectal adenoma. Cancer Prev Res (Phila) 2012;5(6):855-63.

[34] Abuli A, Fernández-Rozadilla C, Giráldez MD, et al. A two-phase case-control study for colorectal cancer genetic susceptibility: candidate genes from chromosomal regions 9q22 and 3q22. BJC 2011;105:870-5.

[35] Uppal S, Diggle CP, Carr IM, et al. Mutations in 15-hydroxyprostaglandin dehydrogenase cause primary hypertrophic osteoarthropathy. Nat Genet 2008;40(6):789-93.

[36] Zhang Z, Xia W, He J, et al. Exome sequencing identifies SLCO2A1 mutations as a cause of Primary Hypertrophic Osteoarthropathy. Am J Hum Genet 2012;90(1):125-32

Table S1. Characterization of genetic polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes and quality control results

Gene	tagSNP	Other SNPs on the block	Genotype call rate (controls/ cases)	Genotype concordance rate	HWE	Passed quality check?
COX-2	rs689466	Candidate gene	0.98 / 0.95	0.97	0.901	Yes
	rs20417	Candidate gene	0.98 / 0.93	0.92	0.998	No
	rs5275	Candidate gene	0.96 / 0.95	1.00	0.999	Yes
HPGD	rs2555639	Candidate gene	0.99 / 0.98	1.00	0.989	Yes
	rs1346271	singleton	1.00 / 0.91	1.00	0.167	Yes
	rs2555632	rs3101255	1.00 / 0.92	1.00	0.681	Yes
	rs2303520	rs13127058	0.99 / 0.94	1.00	0.633	Yes
	rs1863642	rs2612659	1.00 / 0.96	1.00	0.474	Yes
	rs1426945	rs3756273	1.00 / 0.86	0.97	0.976	No
	rs12500316	rs1863641	1.00 / 0.93	1.00	0.508	Yes
		rs11722919				
	rs8752	rs1426947	1.00 / 0.90	1.00	0.948	Yes
		rs2612658				
		rs11133041				
		rs11724251				
	rs2612656	rs1816204	0.94 / 0.91	0.96	0.917	Yes
		rs3857075				
SLCO2A1	rs4241362	rs4241361	1.00 / 0.90	1.00	0.756	Yes
		rs4634113				
		rs6804798				
		rs9828294				
		rs9855403				
		rs9874493				
		rs9882333				
		rs11720811				
	rs7646392	rs4327389	1.00 / 0.78	1.00	0.550	No
		rs4854777				
		rs5013525				
		rs7646298				
		rs7646473				
		rs12695600				
	rs6439448	rs2370512	0.99 / 0.98	0.97	0.979	Yes
		rs3923835				
		rs3923835				
		rs4854768				
		rs4854769				
		rs34550074				
	rs9821091	rs7630191	1.00 / 0.93	1.00	0.651	Yes
		rs9841380				
		rs6439450				
		rs7617777				
		rs9834727				
	rs9820625	rs9836830	1.00 / 0.94	1.00	0.948	Yes
		rs9917636				
		rs11709172				
		rs13083175				
	rs9834412	rs4854785	1.00 / 0.82	1.00	0.951	No
		rs13067921				
	rs4241365	rs7653639	1.00 / 0.94	1.00	0.923	Yes
	rs4331673	rs11720843	1.00 / 0.93	1.00	0.994	Yes
	rs4854784	rs7636169	1.00 / 0.80	0.97	0.871	No
	rs7340717	rs7340718	0.99 / 0.93	1.00	0.600	Yes
	rs7616492	rs10935089	1.00 / 0.98	1.00	0.938	Yes
	rs7625035	rs9822027	1.00 / 0.91	1.00	0.455	Yes
	rs1131598	Singleton	1.00 / 0.96	1.00	0.441	Yes
	rs10935090	Singleton	1.00 / 0.91	1.00	0.815	Yes
	rs11915399	Singleton	1.00 / 0.93	1.00	0.998	Yes
ABCC4	rs4148422	rs17300935	0.99 / 0.92	0.95	0.006	No
	rs9524821	[rs9516532]	0.99 / 0.90	1.00	0.604	Yes
	rs3782958	rs4148515	1.00 / 0.97	1.00	0.931	Yes
		rs10508023				
	rs869951	rs871052	1.00 / 0.92	1.00	0.854	Yes
		rs8001444				

Table S1. Characterization of genetic polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes and quality control results

Gene	tagSNP	Other SNPs on the block	Genotype call rate (controls/ cases)	Genotype concordance rate	HWE	Passed quality check?
ABCC4	rs4771912	rs7981095	1.00 / 0.95	0.97	0.936	Yes
	rs4148421	rs9524864	1.00 / 0.85	1.00	0.998	No
		rs9524873				
		rs10508017				
	rs8002180	rs4148424	1.00 / 0.97	1.00	0.694	Yes
		rs4771910				
		rs7317112				
		rs7322318				
		rs8001475				
		rs9584288				
		rs9590228				
	rs2127295	rs2698243	1.00 / 0.95	0.97	0.657	Yes
		rs1564355				
		rs1617785				
		rs1630807				
		rs1678363				
		rs1678394				
		rs1729748				
		rs2766481				
		rs3825415				
		rs6650282				
	rs1751051	[rs1751050]	1.00 / 0.93	1.00	0.999	Yes
	rs9590220	rs9590216	1.00 / 0.90	0.96	0.018	No
		rs17235152				
	rs2892715	rs9561814	1.00 / 0.95	1.00	0.473	Yes
	rs2892713	rs12865305	1.00 / 0.95	1.00	0.313	Yes
	rs4612933	rs899494	1.00 / 0.93	1.00	0.936	Yes
		rs899495				
		rs899496				
		rs1678403				
		rs1824911				
		rs1824913				
		rs1926657				
		rs3782965				
		rs4148465				
		rs4148469				
		rs4303338				
		rs4334136				
		rs4505186				
		rs4773854				
		rs4773855				
		rs7325019				
		rs7333234				
		rs7335147				
		rs7983336				
		rs7987653				
		rs7988494				
		rs9524831				
		rs9524833				
		rs9524845				
		rs9524856				
		rs12870204				
	rs4148437	rs9556466	1.00 / 0.90	1.00	0.665	Yes
		rs2892716				
		rs4148436				
		rs4148446				
		rs10508018				
	rs1611822	rs1751015	1.00 / 0.96	1.00	0.969	Yes
	rs1678386	rs9516530	1.00 / 0.97	1.00	0.818	Yes
	rs2274403	rs3864997	1.00 / 0.95	0.97	0.862	Yes
		rs4148481				
	rs17268122	rs17268163	0.97 / 0.65	0.96	0.027	No
	rs1751027	rs1564351	0.99 / 0.95	1.00	0.396	No*
		rs4148487				
		rs17189390				
		rs17268170				
	rs17268122	rs17268163	0.97 / 0.65	0.96	0.027	No

Table S1. Characterization of genetic polymorphisms in *COX-2/HPGD/SLCO2A1/ABCC4* genes and quality control results

Gene	tagSNP	Other SNPs on the block	Genotype call rate (controls/ cases)	Genotype concordance rate	HWE	Passed quality check?
ABCC4	rs1751027	rs1564351	0.99 / 0.95	1.00	0.396	No*
		rs4148487				
		rs17189390				
		rs17268170				
	rs4148476	rs4773843	1.00 / 0.93	1.00	0.280	Yes
		rs9524822				
	rs1678374	rs1751025	1.00 / 0.95	1.00	0.964	Yes
	rs1678405	rs2793821	100 / 0.95	1.00	0.722	Yes
		rs6492768				
		rs7330933				
	rs1678396	rs2766482	1.00 / 0.92	1.00	0.537	Yes
	rs1678354	rs1751059	0	-	-	No
	rs1751031	rs931111	1.00 / 0.94	1.00	0.372	Yes
		rs1189444				
		rs1189451				
		rs1189452				
		rs1729747				
		rs2619312				
		rs5016378				
	rs7993878	rs9302040	1.00 / 0.96	0.97	0.346	Yes
		rs9302042				
		rs9302043				
		rs9556455				
		rs9561768				
		rs9561769				
		rs9590168				
		rs10219913				
	rs6492763	rs10508024	1.00 / 0.83	0.97	0.520	No
	rs3742106	rs4148544	1.00 / 0.95	1.00	0.987	Yes
		rs4148549				
		rs4148551				
		rs7330196				
		rs9302039				
		rs9524769				

* minor allele frequency<0.15

Table S2. Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal adenoma onset

SNPs rs	Cases n (%)	Controls n (%)	OR	95% CI	*P* value	aOR	95% CI	*P* value
COX-2								
rs689466								
AA	117 (62.9)	323 (68.4)	1.00	Reference	-	1.00	Reference	-
AG	50 (26.9)	133 (28.2)	1.03	0.67-1.60	0.884	-	-	ns
GG	19 (10.2)	16 (3.4)	3.25	1.53-6.91	0.001	3.33	1.54-7.16	0.002
RM	-	-	3.22	1.53-6.77	0.001	3.23	1.52-6.86	0.002
rs5275								
TT	95 (51.4)	235 (50.9)	1.00	Reference	-	1.00	Reference	-
TC	65 (35.1)	189 (40.9)	0.86	0.58-1.28	0.458	-	-	ns
CC	25 (13.5)	38 (8.2)	1.66	0.92-3.01	0.089	-	-	ns
RM9	-	-	1.78	1.01-3.13	0.045	-	-	ns
HPGD								
rs2555639								
TT	93 (48.7)	216 (45.6)	1.00	Reference	-	1.00	Reference	-
TC	59 (30.9)	209 (44.1)	0.65	0.43-0.99	0.044	0.48	0.28-0.83	0.008
CC	39 (20.4)	49 (10.3)	1.83	1.07-3.11	0.025	-	-	ns
RM	-	-	2.21	1.33-3.65	0.002	2.48	1.36-4.53	0.003
rs2612656								
AA	138 (77.5)	295 (65.6)	1.00	Reference	-	1.00	Reference	-
AG	21 (11.8)	137 (30.4)	0.32	0.17-0.63	0.001	0.19	0.068-0.56	0.002
GG	19 (10.7)	18 (4.0)	2.24	0.99-5.07	0.047	-	-	ns
DM	-	-	0.55	0.32-0.92	0.023	0.47	0.23-0.94	0.033
RM	-	-	2.86	1.27-6.41	0.008	3.20	1.22-8.41	0.018
rs8752								
TT	79 (44.9)	197 (41.2)	1.00	reference	-	1.00	Reference	-
TC	57 (32.4)	219 (45.8)	0.66	0.44-0.97	0.034	-	-	ns
CC	40 (22.7)	62 (13.0)	1.59	0.99-2.56	0.054	-	-	ns
RM	-	-	1.94	1.25-3.02	0.003	1.94	1.09-3.44	0.023
rs1346271								
GG	87 (49.2)	174 (36.2)	1.00	reference	-	1.00	Reference	-
GC	54 (30.5)	246 (51.2)	0.46	0.31-0.68	<0.001	0.45	0.28-0.74	0.002
CC	36 (20.3)	60 (12.5)	1.12	0.68-1.85	0.663	-	-	ns
DM	-	-	0.59	0.41-0.84	0.003	0.55	0.35-0.85	0.008
rs2555632								
TT	124 (68.9)	284 (59.3)	1.00	reference	-	1.00	Reference	-
TC	42 (23.3)	174 (36.3)	0.54	0.36-0.81	0.003	0.51	0.30-0.86	0.012
CC	14 (7.8)	21 (4.4)	1.56	0.77-3.18	0.213	-	-	ns
DM	-	-	0.65	0.45-0.94	0.022	-	-	ns
rs2303520								
GG	124 (67.4)	342 (71.4)	1.00	reference	-	1.00	Reference	-
GA	45 (24.5)	123 (25.7)	1.00	0.67-1.49	0.999	-	-	ns
AA	15 (8.2)	14 (2.9)	2.93	1.37-6.24	0.004	-	-	ns
RM	-	-	2.93	1.38-6.20	0.003	-	-	ns
rs1863642								
GG	110 (58.5)	231 (48.1)	1.00	reference	-	1.00	Reference	-
GT	56 (29.8)	212 (44.2)	0.56	0.38-0.80	0.002	0.48	0.30-0.76	0.002
TT	22 (11.7)	37 (7.7)	1.25	0.70-2.22	0.448	-	-	ns
DM	-	-	0.66	0.47-0.92	0.016	0.55	0.36-0.85	0.007
rs12500316								
CC	122 (67.4)	262 (54.7)	1.00	reference	-	1.00	Reference	-
CT	41 (22.7)	191 (39.9)	0.46	0.31-0.69	<0.001	0.44	0.27-0.72	0.001
TT	18 (9.9)	26 (5.4)	1.49	0.78-2.82	0.221	-	-	ns
DM	-	-	0.59	0.41-0.84	0.003	0.49	0.31-0.78	0.002
RM	-	-	1.92	1.02-3.59	0.039	-	-	ns
SLCO2A1								
rs4241362								
TT	124 (70.4)	333 (69.5)	1.00	reference	-	1.00	Reference	-
TC	28 (15.9)	130 (27.1)	0.58	0.36-0.92	0.019	-	-	ns
CC	24 (13.6)	16 (3.3)	3.99	2.04-7.81	<0.001	3.47	1.58-7.58	0.002
RM	-	-	4.53	2.33-8.80	<0.001	3.90	1.80-8.43	0.001
rs6439448								
CC	160 (83.3)	320 (66.7)	1.00	reference	-	1.00	Reference	-
CA	21 (10.9)	143 (29.8)	0.29	0.18-0.48	<0.001	0.24	0.12-0.49	<0.001
AA	11 (5.7)	17 (3.5)	1.29	0.59-2.81	0.527	-	-	ns
DM	-	-	0.40	0.26-0.61	<0.001	0.38	0.22-0.65	<0.0001

Table S2. Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal adenoma onset

SNPs rs	Cases n (%)	Controls n (%)	OR	95% CI	*P* value	aOR	95% CI	*P* value
rs9821091								
GG	84 (46.2)	180 (37.6)	1.00	reference	-	1.00	Reference	-
GA	48 (26.4)	235 (49.1)	0.44	0.29-0.66	<0.001	0.50	0.31-0.81	<0.001
AA	50 (27.5)	64 (13.4)	1.67	1.07-2.63	0.025	-	-	ns
DM	-	-	0.70	0.50-0.99	0.044	0.62	0.40-0.96	0.033
RM	-	-	2.46	1.62-3.73	<0.001	1.77	1.02-3.06	0.041
rs9820625								
AA	79 (42.9)	141 (29.4)	1.00	reference	-	1.00	Reference	-
AC	70 (38.0)	232 (48.3)	0.54	0.36-0.78	0.001	0.60	0.38-0.98	0.040
CC	35 (19.0)	107 (22.3)	0.58	0.36-0.93	0.023	-	-	ns
DM	-	-	0.55	0.39-0.78	0.001	-	-	ns
rs4241365								
TT	108 (59.0)	282 (58.9)	1.00	reference	-	1.00	Reference	-
TC	52 (28.4)	169 (35.3)	0.81	0.55-1.18	0.274	-	-	ns
CC	23 (12.6)	28 (5.8)	2.14	1.18-3.89	0.011	2.63	1.29-5.37	0.008
RM	-	-	2.31	1.29-4.13	0.004	2.60	1.30-5.22	0.007
rs4331673								
CC	126 (68.1)	332 (69.2)	1.00	reference	-	1.00	Reference	-
CA	54 (29.2)	134 (27.9)	1.06	0.73-1.55	0.755	-	-	ns
AA	5 (2.7)	14 (2.9)	0.94	0.33-2.67	0.909	-	-	ns
rs7340717								
GG	75 (41.2)	204 (42.5)	1.00	reference	-	1.00	Reference	-
GT	68 (37.4)	210 (43.8)	0.88	0.60-1.30	0.529	-	-	ns
TT	39 (21.4)	66 (13.8)	1.59	0.99-2.56	0.055	-	-	ns
rs7616492								
GG	70 (36.5)	202 (42.1)	1.00	reference	-	1.00	Reference	-
GA	80 (41.7)	216 (45.0)	1.07	0.74-1.55	0.727	-	-	ns
AA	42 (21.9)	62 (12.9)	1.99	1.23-3.20	0.005	-	-	ns
RM	-	-	1.92	1.24-2.96	0.003	-	-	ns
rs7625035								
AA	114 (64.4)	278 (57.9)	1.00	reference	-	1.00	Reference	-
AG	48 (27.1)	181 (37.7)	0.65	0.44-0.95	0.026	0.58	0.35-0.94	0.028
GG	15 (8.5)	21 (4.4)	1.74	0.87-3.50	0.115	-	-	ns
RM	-	-	2.02	1.02-4.02	0.041	2.68	1.17-6.11	0.020
rs1131598								
AA	128 (68.4)	274 (57.1)	1.00	reference	-	1.00	Reference	-
AG	43 (23.0)	184 (38.3)	0.50	0.34-0.74	<0.001	0.50	0.34-0.74	0.007
GG	16 (8.6)	22 (4.6)	1.55	0.79-3.05	0.201	-	-	ns
DM	-	-	0.61	0.43-0.87	0.003	-	-	ns
RM	-	-	1.94	1.00-3.79	0.048	2.20	1.01-4.80	0.047
rs10935090								
CC	141 (79.7)	382 (79.7)	1.00	reference	-	1.00	Reference	-
CT	27 (15.3)	90 (18.8)	0.81	0.51-1.30	0.388	-	-	ns
TT	9 (5.1)	7 (1.5)	3.48	1.27-9.530	0.010	5.68	1.43-22.53	0.013
RM	-	-	3.61	1.32-9.85	0.008	5.18	1.33-20.17	0.018
rs11915399								
CC	129 (71.3)	328 (68.5)	1.00	reference	-	1.00	Reference	-
CT	38 (21.0)	137 (28.6)	0.69	0.46-1.05	0.082	-	-	ns
TT	14 (7.7)	14 (2.9)	2.50	1.16-5.40	0.016	-	-	ns
RM	-	-	2.75	1.28-5.89	0.007	-	-	ns
ABCC4								
rs9524821								
GG	65 (36.9)	205 (42.8)	1.00	reference	-	1.00	Reference	-
GA	65 (36.9)	209 (43.6)	0.97	0.65-1.43	0.866	-	-	ns
AA	46 (26.1)	65 (13.6)	2.20	1.38-3.52	0.001	2.15	1.18-3.90	0.012
RM	-	-	2.24	1.46-3.43	<0.001	2.38	1.39-4.09	0.002
rs3782958								
GG	135 (71.1)	336 (70.1)	1.00	reference	-	1.00	Reference	-
GC	48 (25.3)	129 (26.9)	0.93	0.63-1.36	0.697	-	-	ns
CC	7 (3.7)	14 (2.9)	1.24	0.49-3.15	0.644	-	-	ns
rs869951								
GG	86 (48.0)	171 (35.6)	1.00	reference	-	1.00	Reference	-
GC	67 (37.4)	226 (47.1)	0.59	0.40-0.85	0.005	0.58	0.37-0.93	0.026
CC	26 (14.5)	83 (17.3)	0.62	0.37-1.03	0.065	-	-	ns
DM	-	-	0.60	0.42-0.84	0.003	0.60	0.39-0.92	0.018

Table S2. Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal adenoma onset

SNPs rs	Cases n (%)	Controls n (%)	OR	95% CI	*P* value	aOR	95% CI	*P* value
rs4771912								
AA	147 (79.5)	359 (74.6)	1.00	reference	-	1.00	Reference	-
AG	29 (15.7)	112 (23.3)	0.63	0.40-0.99	0.044	-	-	ns
GG	9 (4.9)	10 (2.1)	2.19	0.87-5.50	0.087	-	-	ns
rs8002180								
TT	112 (58.9)	248 (51.8)	1.00	reference	-	1.00	Reference	-
TC	60 (31.6)	188 (39.2)	0.71	0.49-1.02	0.063	0.59	0.37-0.94	0.026
CC	18 (9.5)	43 (9.0)	1.07	0.51-1.68	0.802	-	-	ns
rs2127295								
GG	55 (29.7)	137 (28.7)	1.00	reference	-	1.00	Reference	-
GA	86 (46.5)	247 (51.7)	0.86	0.58-1.28	0.461	-	-	ns
AA	44 (23.8)	94 (19.7)	1.16	0.72-1.86	0.547	-	-	ns
rs1751051								
TT	88 (48.6)	234 (48.8)	1.00	reference	-	1.00	Reference	-
TA	51 (28.2)	202 (42.1)	0.67	0.45-1.00	0.046	-	-	ns
AA	42 (23.2)	44 (9.2)	2.54	1.56-4.14	<0.001	2.24	1.25-4.04	0.007
RM	-	-	2.99	1.88-4.76	<0001	2.75	1.58-4.80	<0.001
rs2892715								
GG	61 (32.8)	173 (36.0)	1.00	reference	-	1.00	Reference	-
GA	84 (45.2)	220 (45.7)	1.08	0.74-1.59	0.685	-	-	ns
AA	41 (22.0)	88 (18.3)	1.35	0.84-2.17	0.210	-	-	ns
rs2892713								
CC	131 (70.4)	337 (70.4)	1.00	reference	-	1.00	Reference	-
CT	40 (21.5)	124 (25.9)	0.83	0.55-1.25	0.372	-	-	ns
TT	15 (8.1)	18 (3.8)	2.14	1.05-4.38	0.033	2.72	1.22-6.06	0.014
RM	-	-	2.25	1.11-4.56	0.022	2.50	1.12-5.58	0.025
rs4612933								
CC	124 (68.5)	315 (65.5)	1.00	reference	-	1.00	Reference	-
CT	41 (22.7)	148 (30.8)	0.70	0.47-1.05	0.084	-	-	ns
TT	16 (8.8)	18 (3.7)	2.25	1.11-4.55	0.021	3.10	1.37-7.03	0.007
RM	-	-	2.49	1.24-4.99	0.008	3.03	1.35-6.79	0.007
rs4148437								
TT	78 (44.3)	194 (40.4)	1.00	reference	-	1.00	Reference	-
TC	76 (43.2)	215 (44.8)	0.88	0.61-1.28	0.512	-	-	ns
CC	22 (12.5)	71 (14.8)	0.78	0.45-1.35	0.376	-	-	ns
rs1611822								
CC	66 (35.1)	182 (37.9)	1.00	reference	-	1.00	Reference	-
CT	75 (39.9)	225 (46.9)	0.91	0.62-1.34	0.647	-	-	ns
TT	47 (25.0)	73 (15.2)	1.77	1.11-2.80	0.015	-	-	ns
RM	-	-	1.85	1.23-2.80	0.003	-	-	ns
rs1678386								
AA	111 (58.7)	243 (50.6)	1.00	reference	-	1.00	Reference	-
AC	60 (31.7)	193 (40.2)	0.68	0.47-0.98	0.039	-	-	ns
CC	18 (9.5)	44 (9.2)	0.90	0.50-1.62	0.715	-	-	ns
rs2274403								
AA	55 (30.6)	120 (25.0)	1.00	reference	-	1.00	Reference	-
AG	75 (41.7)	234 (48.8)	0.70	0.46-1.06	0.088	-	-	ns
GG	50 (27.8)	126 (26.2)	0.87	0.55-1.37	0.537	-	-	ns
rs4148476								
TT	126 (69.6)	339 (70.6)	1.00	reference	-	1.00	Reference	-
TG	40 (22.1)	123 (25.6)	0.88	0.58-1.32	0.524	0.57	0.32-0.99	0.046
GG	15 (8.3)	18 (3.8)	2.24	1.10-4.58	0.024	2.81	1.22-6.47	0.015
RM	-	-	2.32	1.14-4.71	0.017	3.22	1.41-7.36	0.005
rs1678374								
TT	82 (44.3)	164 (34.2)	1.00	reference	-	1.00	Reference	-
TC	76 (41.1)	235 (49.1)	065	0.45-0.94	0.021	0.62	0.39-0.98	0.042
CC	27 (14.6)	80 (16.7)	0.68	0.40-1.12	0.131	-	-	ns
DM	-	-	0.66	0.46-0.93	0.017	-	-	ns
rs1678405								
TT	111 (59.7)	199 (41.4)	1.00	reference	-	1.00	Reference	-
TC	69 (37.1)	227 (47.2)	0.54	0.38-0.77	0.001	0.47	0.30-0.74	0.001
CC	6 (3.2)	55 (11.4)	0.20	0.08-0.47	<0.001	0.10	0.02-0.45	0.002
DM	-	-	0.47	0.34-0.67	<0.001	0.41	0.27-0.63	<0.001
RM	-	-	0.26	0.11-0.61	0.001	0.15	0.04-0.63	0.010

Table S2. Genotype frequencies among cases and controls and risk estimates for the involvement of *COX-2/HPGD/SLCO2A1/ABCC4* polymorphisms in colorectal adenoma onset

SNPs rs	Cases n (%)	Controls n (%)	OR	95% CI	*P* value	aOR	95% CI	*P* value
rs1678396								
TT	66 (36.9)	147 (30.6)	1.00	reference	-	1.00	Reference	-
TC	72 (40.2)	248 (51.7)	0.65	0.44-0.96	0.028	-	-	ns
CC	41 (22.9)	85 (17.7)	0.75	0.52-1.08	0.116	-	-	ns
rs1751031								
AA	133 (72.7)	299 (62.3)	1.00	reference	-	1.00	Reference	-
AG	35 (19.1)	166 (34.6)	0.47	0.31-0.72	<0.001	0.53	0.32-0.88	<0.001
GG	15 (8.2)	15 (3.1)	2.24	1.06-4.72	0.030	-	-	ns
DM	-	-	0.62	0.43-0.90	0.012	-	-	ns
RM	-	-	2.76	1.32-5.78	0.005	2.99	1.11-8.00	0.030
rs7993878								
GG	135 (71.8)	361 (75.1)	1.00	reference	-	1.00	Reference	-
GA	38 (20.2)	107 (22.2)	0.95	0.62-1.44	0.809	-	-	ns
AA	15 (8.0)	13 (2.7)	3.34	1.53-7.32	0.002	-	-	ns
DM	-	-	3.38	1.55-7.37	0.001	3.14	1.09-9.01	0.033
rs3742106								
AA	67 (36.2)	166 (34.6)	1.00	reference	-	1.00	Reference	-
AC	82 (44.3)	234 (48.8)	0.87	0.59-1.27	0.465	-	-	ns
CC	36 (19.5)	80 (16.7)	1.12	0.69-1.81	0.660	-	-	ns

aOdds Ratio, Logistic regression (Forward:conditional model) including gender, smoking habits and age (median global age of 59 years used as cutoff). CI, confidence interval

CHAPTER VI: INFLUENCE OF GENETIC POLYMORPHISMS IN PROSTAGLANDIN E_2 (PGE_2) PATHWAY ON mRNA EXPRESSION OF *COX-2*, *HPGD*, *ABCC4* AND *SLCO2A1* GENES IN COLORECTAL TUMORS.

INFLUENCE OF GENETIC POLYMORPHISMS IN PROSTAGLANDIN E_2 (PGE_2) PATHWAY ON mRNA EXPRESSION OF *COX-2, HPGD, ABCC4* AND *SLCO2A1 GENES* IN COLORECTAL TUMORS.

Carina Pereira[1,2], Joana Ribeiro[1], Sara Queirós[1], Luís Lima[3], Hugo Sousa[1], Ana Galaghar[4], Pedro Pimentel-Nunes[5,6], Catarina Brandão[5], Luís Moreira-Dias[5], Rui Medeiros[1,2,7,8] Mário Dinis-Ribeiro[5,9]

[1]Molecular Oncology Group, Investigation Centre, Portuguese Institute of Oncology, Porto, Portugal; [2]Abel Salazar Institute of Biomedical Sciences, University of Porto, Porto, Portugal; [3]Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology, Porto, Portugal; [4]Department of Pathology, Portuguese Institute of Oncology, Porto, Portugal; [5]Gastroenterology Department, Portuguese Institute of Oncology, Porto, Portugal; [6]Physiology Department, Faculty of Medicine, University of Porto, Porto, Portugal; [7]CEBIMED, Faculty of Health Sciences of Fernando Pessoa University, Porto, Portugal; Research Department, [8]Portuguese League Against Cancer, Porto, Portugal; [9]Faculty of Medicine, CINTESIS/Department of Biostatistics and Medical Informatics, University of Porto, Porto, Portugal

Correspondence to:

Carina Pereira

Molecular Oncology Group - IPOP Research Centre

Portuguese Institute of Oncology - Porto,

Rua Dr. Bernardino de Almeida

4200-072 Porto, Portugal.

Tel: +351 22 508 4000 (5115); fax: + 351 22 508 4001

e-mail: anacmpereira@gmail.com

Keywords:

Colorectal cancer; Colorectal adenomas; Genetic polymorphisms; *COX-2*; *HPGD*; *SLCO2A1*; *ABCC4*; mRNA expression

ABSTRACT

Deregulation of the COX-2/PGE_2 pathway is an early event in colorectal carcinogenesis leading to the stimulation of a plethora of oncogenic pathways with repercussion on most if not all hallmarks of cancer. Our group has previously identified several genetic variants in the COX-2/PGE_2 pathway has risk biomarkers for the occurence and recurrence of colorectal tumors.

A functional study was design to assessed the influence of polymorphisms on *COX-2*, *HPGD*, *SLCO2A1* or *ABCC4* mRNA levels on colonic tissues from 60 patients diagnosed with CRC, through Real-time PCR.

Thirteen out of the 20 genetic variants analysed presented and allelic-specific expression profile. The rs689466GG genotype was associated with a 7-fold overexpression of *COX-2* in CRC tissues (-1.57±0.10 vs-4.42±1.58 for the AA genotype, *P*=0.024). Similarly, the heterozygous genotype for the rs1425945 polymorphism exhibited a trend for higher levels of *HPGD* (-1.10±1.41 vs -2.02±2.18 for the GG homozygous genotype, *P*=0.163). An approximately 2-fold overexpression was reported for AC and CC genotypes of rs9820625 tagSNP when compared with the AA homozygous genotype in normal-appearing mucosa (-4.96±1.78 and -5.17±1.28 vs -6.08±0.96, respectively). Furthemore,the *ABCC4* levels were progressively lower in rs1678405TC and CC genotypes in malignant mucosa (-2.26±1.47 and -3.39±0.92, respectively, vs -1.43±1.18 with the TT genotype, *P*=0.049).

The present functional study reports the involvement of genetic variants on *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* genes' expression providing a biological reasoning underlying the epidemiological data.

INTRODUCTION

A large number of common low-penetrance genetic variants each exhibiting a small influence on the risk are expected to be involved on the occurrence of colorectal tumors [1]. Furthermore, studies uncovering the genetic basis of variation in gene expression, also known as, expression qualitative trait loci (eQTL) have implicated polymorphisms as regulators of the expression of genes with considerable variation [2].

A large body of epidemiological and functional data as highlighted the central role of COX-2/PGE_2 pathway in colorectal carcinogenesis [3-7]. Moreover, the nonsteroidal anti-inflammatory drugs target the cyclooxygenases (COX) enzymes to exert their preventive effects on colorectal tumors development [8].

COX-2, the inducible isoenzyme, was shown to be up-regulated by 2 to 50-fold in human colorectal adenomas and adenocarcinomas [9]. This deregulation increases the synthesis of prostaglandin E_2 (PGE_2) that once carried-out across the membrane *via* the multidrug resistance-associated protein 4 (MRP4) triggers several oncogenic signaling pathways that contribute to most of the known hallmarks of cancer, including inhibition of apoptosis, stimulation of cell proliferation and angiogenesis [10-12]. The first step in PGE_2 catabolism requires its transport back to the cytoplasm from the extracellular microenvironment by the specific PG transporter (PGT), where NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) oxidizes PGE_2 to yield inactive 15-keto metabolites [13].

Backlund and colleagues [14] reported a repressed expression and activity of 15-PGDH in human CRC and $Apc^{Min/+}$ mouse adenoma resulting in a decreased catabolism of PGE_2. The down-regulation of 15-PGDH in colorectal tumors was corroborated in subsequent studies [15,16]. Further exploring this pathway Holla and colleagues [17], showed that PGT expression was also decreased whereas the MRP4 was elevated in CRC. In fact, mRNA levels of PGT and MRP4 are inversely regulated in human CRC compared to normal mucosa and in intestinal adenomas from $Apc^{Min/+}$ mice [17]. Deregulation of these molecules leads to higher levels of PGE_2 extracellularly thus potentiating the effects of COX-2/PGE_2 pathway [12].

Our group previously identified several genetic polymorphisms in the genes coding for COX-2, 15-PGDH (*HPGD, hydropxyprostaglandin dehydrogenase*), PGT (*ABCC4, ATP-binding cassete sub-family c member 4*) and MRP4 proteins (*SLCO2A1*, *solute carrier organic anion transporter family, member 2A1*) as risk markers not only in the development of colorectal cancer (CRC) and early stage colorectal tumors but also in the occurrence of metachronous adenomatous polyps in a Northern Portuguese population [18,19]. The scarcity of available data evaluating the molecular relevance of these polymorphisms, prompted us to investigate the functional impact of the genetic variability in *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* on genes' expression to provide a biological plausibility underlying the epidemiological associations [20].

MATERIAL AND METHODS

Patient samples

Sixty patients were selected from a subset of 129 CRC patients from the original observational study [18], recruited between 2002 and 2007 at the Portuguese Institute of Oncology of Porto, based on the availability of formalin fixed paraffin embedded (FFPE) tissues. Only patients not submitted to treatments with chemotherapy and/or radiotherapy prior to surgical resection of tumor were included. The description of patients is displayed in Table 1.

In comparison with the entire group, tumors from these CRC patients tended to have lower stages (70.6% vs 52.6) and be located preferentially at the colon (61.0 vs 47.7).

Table 1. Description of patients with colorectal cancer

	CRC mucosa (n=39)	Normal mucosa (n=60)	*P* value
Age (years)			
Mean (SD)	65 (7.39)	65 (7.19)	0.977
Median (min-max)	66 (50-75)	66 (50-75)	
Gender, n (%)			
Male	21 (53.8)	36 (60.0)	0.545
Female	18 (46.2)	24 (40.0)	
Smoking habits, n (%)			
Never-smokers	11 (73.3)	12 (75.0)	0.916
Ever-smokers	4 (26.7)	4 (25.0)	
Tumor location, n (%)			
Rectum	36 (61.0)	-	
Colon	23 (39.0)	-	
Stage, n (%)			
I-II	24 (70.6)	-	
III-IV	10 (29.4)	-	

Polymorphisms selection and genotyping

The selection of polymorphisms and the genotype characterization is described elsewhere [18]. Briefly, 55 tagSNPs were included in the case-controls studies after being retrieved from a set of common SNPs in the Caucasian population of HapMap project (minor allele frequency: ≥0.15%; r^2<0.8). In this study we only addressed the genetic variations that were identified as susceptibility markers for the development or recurrence of colorectal tumors in the observational studies (Table S1 of supplementary data).

The tagSNPs genotyping was performed using MassARRAY iPLEX Gold technology based on multiplex amplification followed by mass-spectrometric product separation (Sequenom, San Diego, CA). The rs689466 in *COX-2* gene was characterized by Real-Time PCR using validated TaqMan SNP genotyping assay (C___2517145_20).

RNA isolation and quantification

RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues using the Absolutely RNA FFPE Kit following the manufacture's instructions (Stratagene, La Jolla, CA, USA). The number of 10μm thickness sections used for RNA extraction varied from two to four, depending on the size of tumor area. A 3μm section was stained with hematoxylin and eosin (H&E) and a histopathological characterization was rendered, by a senior pathologist. The enriched tumor cell

area were microdissected into a 1.5 ml reaction tube containing 1 ml of deparaffinization reagent (d-limonene) by scratching using a sterile single-use scapel. Normal colon tissues were prepared from the surgical margins at the edge of a colon resection. RNA was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA quality was determined by measuring the optical density (OD) 260/280 ratio.

Reverse transcriptase and mRNA quantification

Up to 2µg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA). Briefly, in a 20µl reaction mix, 2.0µl of 1X RT buffer, 0.8µl 25X dNTP mix, 2.0µl 10X RT random primers and 1.0µl of Multiscribe Reverse Transcriptase were used. The amplification conditions were as follows: annealing at 25ºC for 10 min, extension at 37ºC for 120 min and RT inactivation at 85ºC for 5 min. All reverse transcriptase reactions included two no-template negative controls. Furthermore, the QPCR Human Reference Total RNA, included in the RNA extraction kit was included as a positive control to monitor the quality of RT reaction.

Real-time PCR amplification of cDNA was performed in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using 10µl of 2X TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 1µl of Gene Expression Assay and 30 to 40ng of cDNA template. The mRNA expression of *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* genes was measured with the gene expression assays Hs00153133_m1, Hs00960586_g1, Hs00194554_m1, Hs00988717_m1, respectively (Applied Biosystems). Thermal cycling for all assays was 95ºC for 10 min, followed by 50 cycles of 95ºC for 15 sec and 60ºC for 1 min. Cytokeratin 20 (KRT20) was used as the reference gene for normalization and was amplified using the human hydrolysis Probe/Primer assay Hs00300643_m1 from Applied Biosystems. *KRT20* is a specific marker for colonic epithelial mass with previously reported uniform expression by microarray across colon tissue biopsies [20,21].

The endpoint of the qPCR data was the cycle threshold (*C*t) determined as the average values from two independent real-time PCR reactions. The relative expression of mRNA was defined as the difference between Ct*s* of the

amplification curves of the target genes and the KRT20 reference gene (-ΔCt). The fold difference in expression was determined following the Livak method, also known as $2^{-\Delta\Delta Ct}$ [22].

Quality control

All reverse transcriptase reactions included two no-template negative controls. Furthermore, the QPCR Human Reference Total RNA included in the RNA extraction kit was used as a positive control in the RT reaction. The efficiency of the amplification reaction for each Probe/Primer was calculated, also as the limit of detection. Efficiency superior to 95% and sensitivity above 20ng was reported for all expression assays.

Data analysis

Statistical analysis was performed using the computer software *IBM® SPSS® (Statistical Package for Social Sciences) Statistics* version 20.0 for Mac. Student's t-test was used to compare the expression between cancer and normal mucosa. The difference in mean tissue expression between the three possible genotypes was evaluated using a one-way ANOVA. The non-parametric corresponding statistical tests were applied when appropriate.

RESULTS

We were able to successfully extract RNA from normal-appearing mucosa in all 60 cases and CRC tissues from 39 patients. The mean expression of KRT20 was 31.17±2.14 with a 1.36-fold variation (lowest to highest), suggesting a good stability across samples and lesions. The mean mRNA expression values are displayed in Table 2. Overall, the *COX-2* gene was found to be overexpressed in CRC tumors (-3.99±1.64 vs -5.22±1.67 in normal-appearing mucosa, P=0.001), leading to a 2.34-fold increase in mRNA expression, as can be observed in Figure 1. This increased levels in COX-2 was only evident in males (P<0.001)

Table 2. Mean expression values (-ΔCt) for *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* stratified by gender and SNPs

	Normal mucosa					CRC				
	N	Mean expression (SD)	*P** value	*P* value	Fold-difference**	N	Mean expression (SD)	*P** value	*P* value	Fold-difference**
COX-2	57	-5.22 (1.67)	-	-	-	37	-3.99 (1.64)	-	**0.001**	**2.34**
Gender										
Female	22	-4.54 (1.60)	-	-	-	17	-4.43 (1.44)	-	0.821	1.08
Male	33	-5.73 (1.60)	-	-	-	18	-3.58 (1.67)	-	**<0.001**	**4.44**
rs689466										
AA	26	-4.99 (1.74)		-		21	-4.42 (1.58)		-	
AG	26	-5.32 (1.58)	0.155	0.481	0.80	13	-3,50 (1.40)	0.024	0.095	1.89
GG	3	-6.96 (1.64)		0.073	0.26	2	-1,57 (0.10)		**0.021**	**7.21**
HPGD	60	-1.59 (1.49)	-	-	-	39	-1.64 (1.97)	-	0.848	0.96
Gender										
Females	24	-1.47 (1.07)	-	-	-	18	-1.90 (1.63)	-	0.647	0.74
Males	36	-1.66 (1.76)	-	-	-	20	-1.66 (2.20)	-	0.986	1.00
rs12500316										
CC	38	-1.38 (1.76)		-	-	29	-1.69 (1.89)		-	-
CT	14	-1.95 (0.99)	0.193	0.087	0.67	4	-0.87 (1.90)	0.449	0.423	1.76
TT	6	-1.79 (0.80)		0.374	0.75	4	-2.70 (3.12)		0.360	0.50
rs1863642										
GG	32	-1.11 (1.53)		-	-	22	-1.66 (1.99)		-	-
GT	21	-2.19 (1.49)	0.035	**0.015**	**0.47**	12	-1.36 (1.64)	0.486	0.656	1.23
TT	6	-1.90 (0.74)		0.227	0.58	4	-2.76 (3.08)		0.357	0.47
rs1346271										
GG	29	-1.34 (1.78)		-	-	21	-2.05 (1.70)		-	-
GC	15	-2.04 (1.11)	0.582	**0.039**	**0.62**	6	-0.88 (1.42)	0.313	0.136	2.27
CC	16	-1.60 (1.23)		0.148	0.84	12	-1.45 (2.56)		0.428	1.51
rs1425945										
GG	35	-1.33 (1.50)		-	-	25	-2.02 (2.18)	-	-	-
GA	23	-1.96 (1.81)	0.319	0.130	0.65	14	-1.10 (1.41)	-	0.163	1.89
AA	2	-1.79 (0.79)		0.608	0.73	0	-	-	-	-
rs2555639										
TT	34	-1.33 (1.49)		-	-	24	-1.67 (2.26)		-	-
TC	19	-2.03 (1.70)	0.216	0.091	0.62	11	-2.16 (1.21)	0.482	0.505	0.71
CC	7	-1.59 (0.84)		0.665	0.84	3	-1.10 (1.19)		0.672	1.48
C carriers	26	-1.91 (1.52)		0.101	0.67	14	-1.94 (1.24)		0.692	0.83

Table 2. Mean expression values (-ΔCt) for *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* stratified by gender and SNPs

	Normal mucosa					CRC				
	N	Mean expression (SD)	*P** value	*P* value	Fold-difference**	N	Mean expression (SD)	*P** value	*P* value	Fold-difference**
SLCO2A1	58	-5.30 (1.46)	-	-	-	39	-4.26 (1.63)	-	0.001	2.06
Gender										
Males	32	-5.28 (1.56)	-	-	-	20	-4.29 (1.53)	-	**0.011**	**1.99**
Females	24	-5.31 (1.40)	-	-	-	18	-4.26 (1.83)	-	**0.029**	**2.07**
rs6439448										
CC	39	-5.19 (1.62)		-	-	27	-4.32 (1.49)		-	-
CA	11	-5.43 (1.30)	0.774	0.598	0.85	8	-4.49 (2.05)	0.176	0.790	0.89
AA	3	-5.20 (0.46)		0.678	0.99	3	-2.51 (1.57)		0.058	3.51
rs1131598										
AA	29	-5.32 (1.71)		-	-	20	-4.16 (2.62)		-	-
AG	21	-5.21 (1.76)	0.718	0.510	1.08	11	-4.02 (1.99)	0.518	0.830	1.10
GG	6	-5.38 (0.88)		0.507	0.96	7	-4.91 (1.27)		0.280	0.59
rs7616492										
GG	19	-5.52 (1.10)		-	-	17	-4.52 (1.45)		-	-
GA	25	-5.17 (1.73)	0.847	0.731	1.27	13	-4.78 (1.61)	0.701	0.210	0.84
AA	10	-5.11 (1.62)		0.425	1.33	9	-4.34 (2.07)		0.790	1.13
rs9820625										
AA	14	-6.08 (0.96)		-	-	10	-4.31 (2.06)		-	-
AC	24	-4.96 (1.78)	0.024	**0.032**	**2.17**	17	-4,04 (1.40)	0.640	0.681	1.20
CC	16	-5.17 (1.28)		**0.030**	**1.88**	10	-4.68 (1.79)		0.677	0.77
rs7340717										
GG	28	-5.35 (1.21)		-	-	18	-4.15 (1.83)		-	-
GT	17	-5.03 (2.00)	0.272	0.515	1.25	13	-4.17 (1.37)	0.965	0.973	1
TT	9	-6.01 (0.74)		0.131	0.63	5	-4.37 (1.48)		0.812	0.86
ABCC4	60	-3.03 (1.36)	-	-	-	38	-1.74 (1.33)	-	**<0.001**	**2.44**
Gender										
Males	35	-3.36 (1.41)	-	-	-	20	-1.74 (1.40)	-	**0.011**	**2.44**
Females	23	-2.55 (1.23)	-	-	-	18	-1.74 (1.29)	-	**0.029**	**1.75**
rs9524821										
GG	19	-3.05 (1.62)		-	-	12	-1.67 (1.43)		-	-
GA	26	-3.09 (1.26)	0.752	0.408	0.97	15	-1.32 (1.25)	0.603	0.573	1.27
AA	11	-2.87 (1.45)		0.800	1.13	10	-1.41 (1.43)		0.674	1.20

Table 2. Mean expression values (-ΔCt) for *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* stratified by gender and SNPs

	Normal mucosa					CRC				
	N	Mean expression (SD)	*P** value	*P* value	Fold-difference**	N	Mean expression (SD)	*P** value	*P* value	Fold-difference**
rs1751051										
TT	29	-2.98 (1.13)		-	-	22	-1.61 (1.24)		-	-
TA	19	-3.49 (1.75)	0.138	0.223	0.70	8	-1.64 (1.19)	0.987	0.946	0.98
AA	7	-2.28 (1.28)		0.163	1.62	6	-1.70 (1.58)		0.877	0.94
T carriers	48	-3.18 (1.41)		0.118	1.87	30	-1.62 (1.21)		0.694	0.95
rs1678405										
TT	35	-3.10 (1.30)		-	-	24	-1.43 (1.18)		-	-
TC	19	-2.86 (1.64)	-	0.380	1.18	10	-2.26 (1.47)	0.049	0.900	0.56
CC	1	-		-	-	2	-3.39 (0.92)		0.032	0.26
rs1751031										
AA	40	-2.77 (1.30)		-	-	25	-1.60 (1.32)		-	-
AG	12	-3.83 (1.44)	0.053	**0.019**	**0.48**	6	-1.50 (1.18)	0.320	0.859	1.07
GG	5	-3.46 (1.54)		0.380	0.62	7	-2.43 (1.42)		0.162	0.56
rs1678396										
TT	25	-2.82 (1.51)		-	-	18	-1.63 (1.32)		-	-
TC	21	-3.16 (1.51)	0.507	0.449	0.79	12	-1.88 (1.31)	0.879	0.607	0.84
CC	11	-3.37 (0.75)		0.263	0.68	8	-1.78 (1.54)		0.807	0.90
rs2274403										
AA	19	-3.13 (1.47)		-	-	14	-1.84 (1.37)		-	-
AG	25	-3.14 (0.96)	0.686	0.965	0.99	15	-1.81 (1.42)	0.708	0.958	1.02
GG	13	-2.74 (1.97)		0.538	1.31	7	-1.33 (1.35)		0.437	1.42
rs3742106										
AA	24	-3.38 (1.27)		-	-	14	-2.21 (1.24)		-	-
AC	29	-2.80 (1.47)	0.286	0.133	1.49	20	-1.52 (1.14)	0.235	0.101	1.61
CC	4	-2.70 (1.47)		0.388	1.60	4	-1.22 (2.27)		0.258	1.99
rs6492763										
TT	28	-2.84 (1.24)		-	-	19	-1.66 (1.47)		-	-
TC	20	-3.11 (1.52)	0.612	0.504	0.83	10	-1.62 (1.07)	0.831	0.937	1.03
CC	7	-3.82 (1.72)		0.362	0.51	8	-1.98 (1.46)		0.612	0.80
rs869951										
GG	24	-3.36 (1.52)		-	-	14	-2.39 (0.93)		-	-
GC	22	-3.54 (1.34)	0.335	0.346	0.88	13	0.74 (1.33)	0.003	**0.001**	**8.75**
CC	11	-2.57 (1.17)		0.152	1.73	9	-1.98 (1.21)		0.366	1.33
C carriers	33	-2.82 (1.28)		0.157	1.45	22	-1.25 (1.40)		**0.011**	**2.20**

SNP, single nucleotide polymorphism; CRC, Colorectal cancer; SD, standart deviation. At bold, statistical significant results
*calculated using One-way ANOVA or Kruskal-Wallis test, when appropriate;; **calculated using the Livak method ($2^{-\Delta\Delta Ct}$)

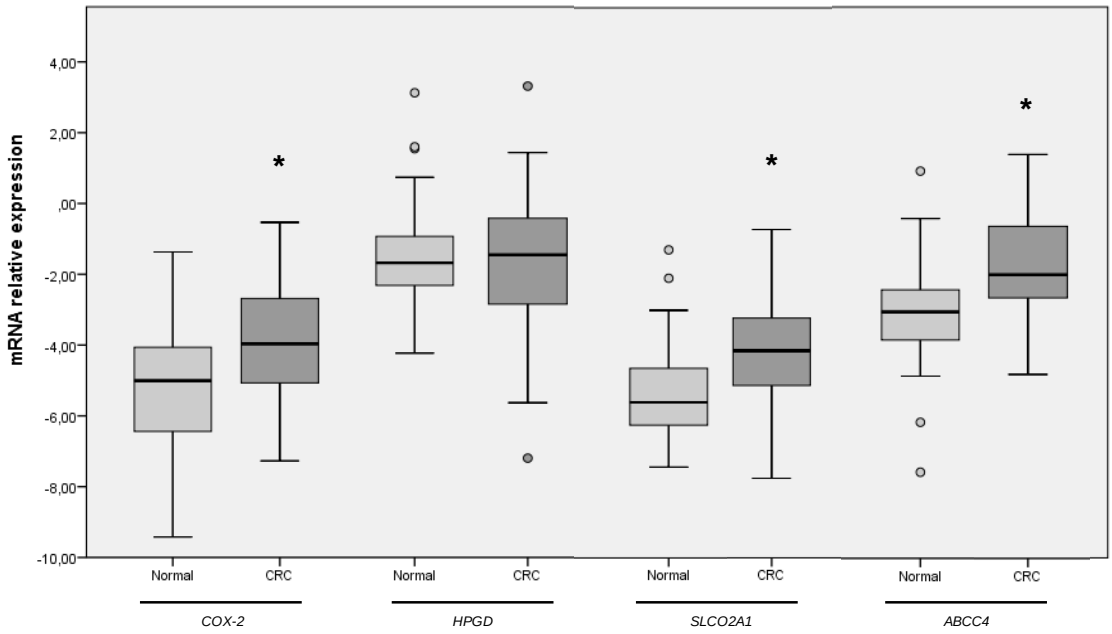


Figure 1. Real-time analysis for *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* mRNA levels (-ΔCT) in normal-appearing mucosa and colorectal cancer (CRC) tissues. The Box plots represent in the horizontal bar the mean value for mRNA in each group. The whiskers represent the maximum and minimum values of the data. Black dots represent outliers. **P*<0.05.

Although detected at high levels in colonic mucosa, no differences in *HPGD* mRNA expression was noticed (-1.59±1.49 vs -1.64±1.97, *P*=0.848). Surprisingly, a 2-fold increase in *SLCO2A1* levels was observed independently of gender (-5.30±1.46 vs -4.26±1.63 in normal mucosa, *P*=0.011). Similarly, the *ABCC4* gene was found at higher levels in CRC (-3.03±1.36 vs -1.74±1.33, *P*<0.001, 2.4 folincrease). No gender-specific behavior was noticed.Considering the high variance in genes' levels, we then questioned if the genetic variants could, if fact, be determinants of mRNA expression?

Thirteen out of the twenty polymorphisms analyzed appear to be involved in gene´s regulation, some of which with a gender and histological-type dependent behavior.

As can be observed in Figure 2, an increasingly overexpression of *COX-2* mRNA is observed from genotypes carrying one to both copies of rs689466G allele in contrast with the AA homozygous genotype (-3.50±1.40, -1.57±0.10 and -4.42±1.58, respectively; *P*=0.024). The rs689466AG and GG genotypes were

further associated with a nearly 2- and 7-fold increase in *COX-2* mRNA expression in colorectal neoplasia. This seems particularly relevant in males. The heterozygous genotype in males' tumors led to an increase in mRNA levels by 3-fold (-2.87±1.34 vs -4.45±1.56 for the AA genotype, *P*=0.041 in males and -4.52±0.79 vs -4.40±1.67, for the AG and AA genotypes, respectively, *P*=0.884 in females). Antagonically, in normal-appearing colonic mucosa, the GG genotype decreased the expression of *COX-2* in comparison with the AA genotype (-6.96±1.64 vs -4.99±1.74, *P*=0.073).

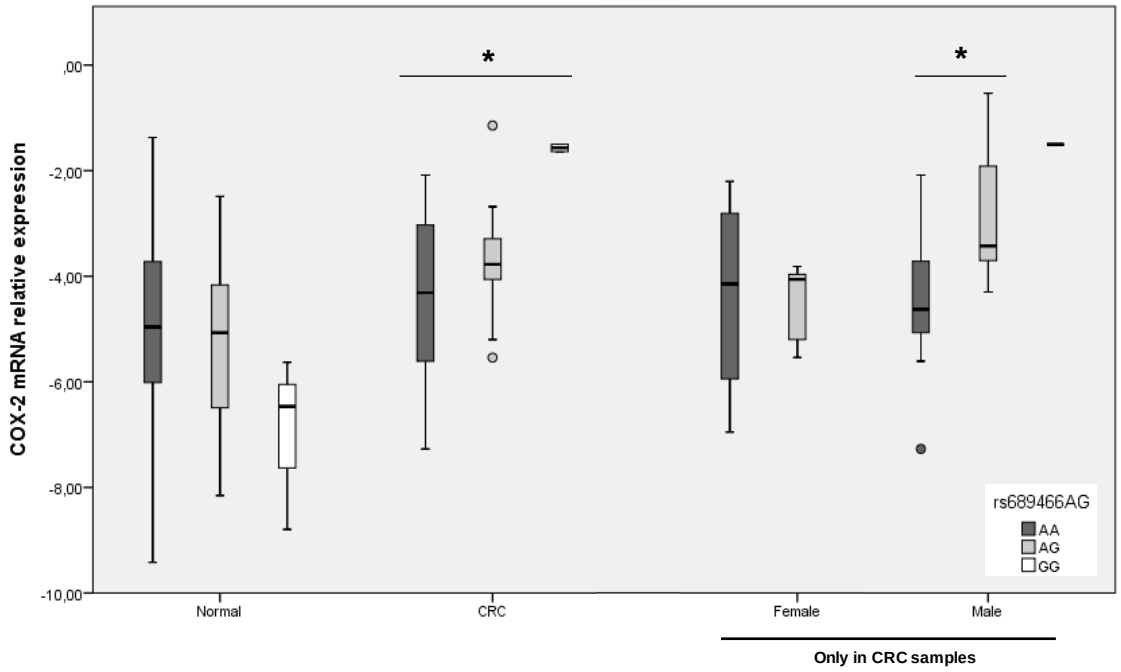


Figure 2. Relative levels of *COX-2* mRNA (-ΔCT) considering the genotypes for the rs689466A>G polymorphism. The Box plots represent in the horizontal bar the mean value for mRNA in each group. The whiskers represent the maximum and minimum values of the data. Black dots represent outliers. **P*<0.05.

Similarly, the GC genotype of rs1346271 polymorphism decreased the levels of *HPGD* mRNA in normal-appearing mucosa (-2.04±1.11 vs -1.11±1.53, *P*=0.039 for GG genotype) and was associated with a 2-fold higher expression in CRC mucosa, although not statistically significant (-0.88±1.42 vs -2.05±1.70, *P*=0.136), as observed in Figure 3. Three other tagSNPs in *HPGD* presented an allele-specific regulation. In normal-appearing mucosa the GC genotype of rs1863642 and TC

heterozygous genotype of rs2555639 polymorphism had a lower mRNA expression in opposition with the respective dominant genotypes (-2.19±1.49 vs -1.11±1.53 for the rs1863642GG genotype, *P*=0.015; and -2.03±1.70 vs -1.33±1.49 for the homozygous TT genotype of rs2555639, *P*=0.091). A trend for a higher expression was noticed with the rs1425945GA genotype in malignant mucosa (-1.10±1.41 vs -2.02±2.18 for GG genotype, *P*=0.163).

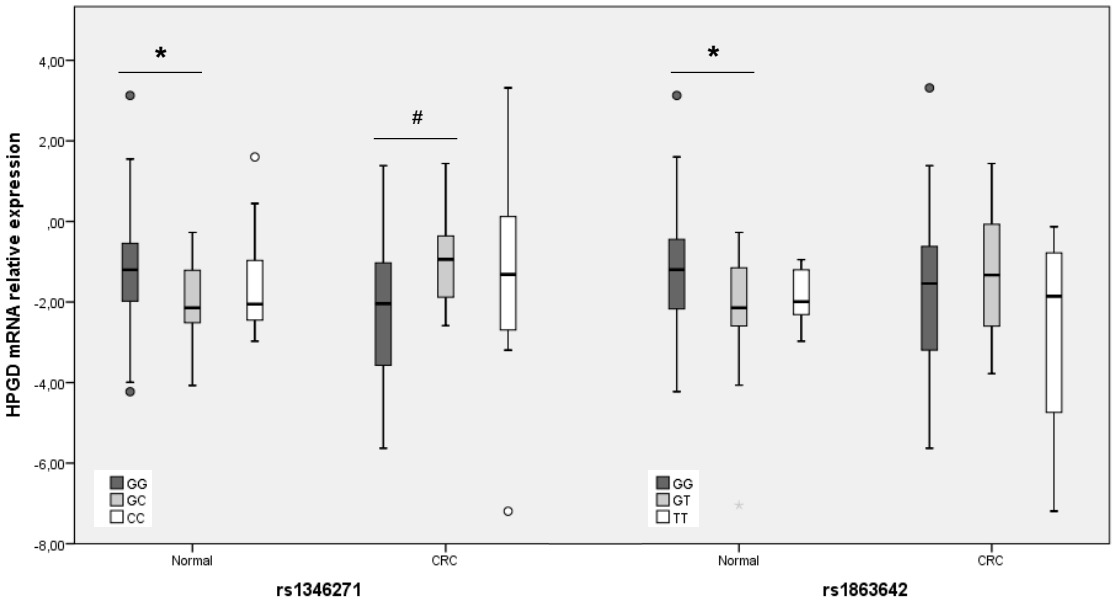


Figure 3. Relative levels of *HPGD* mRNA (-ΔCT) considering the genotypes for the rs1346271G>C (left side) and rs1863642G>T (right side) polymorphisms. The Box plots represent in the horizontal bar the mean value for mRNA in each group. The whiskers represent the maximum and minimum values of the data. Black dots represent outliers. **P*<0.05; $^{\#}$*P*=0.136.

Considering the *SLCO2A1* gene, an approximately 2-fold overexpression was reported for AC and CC genotypes of rs9820625 tagSNP when compared with the AA homozygous genotype in normal-appearing mucosa (-4.96±1.78 and -5.17±1.28 vs -6.08±0.96, respectively (see Figure 4). Additionally, two trends were observed with the rs6439448C>A and rs7340717G>T in CRC tissues and mucosa with no observable tumors, respectively. An enhanced expression was evident with the AA genotype of rs6439448 (-2.51±1.57 vs -4.32±1.49 for the CC genotype, *P*=0.058) in opposition with the rs73407171TT genotype that downregulated *SLCO2A1* mRNA levels (-6.01±0.74 vs -5.23±1.54 observed for G allele carriers, *P*=0.107).

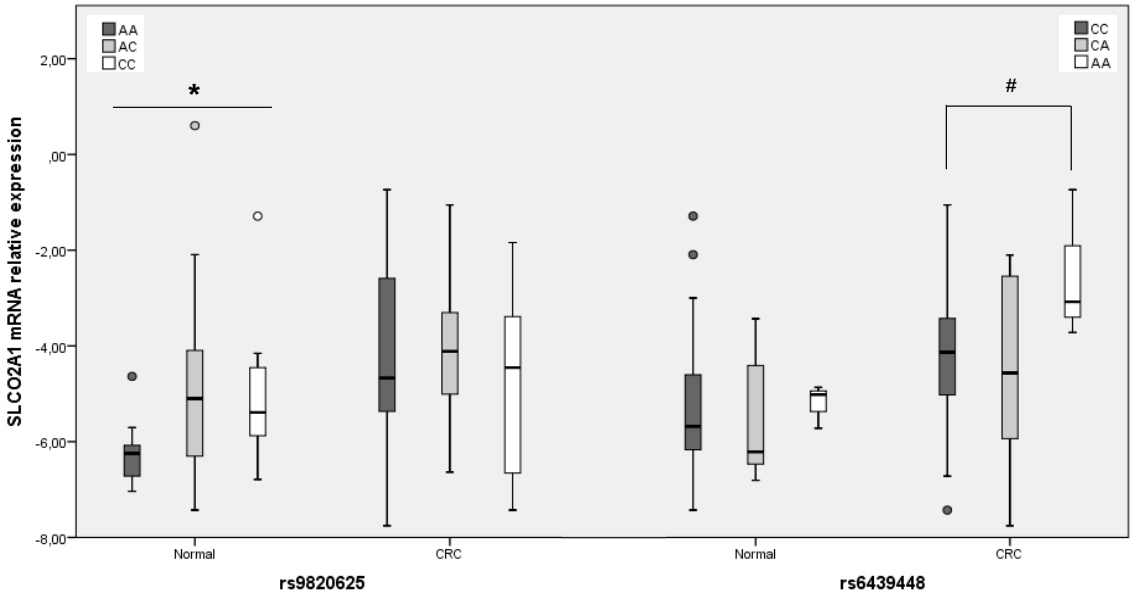


Figure 4. Relative levels of *SLCO2A1* mRNA (-ΔCT) considering the genotypes for the rs9820625A>C (left side) and rs6439448C>A (right side) polymorphisms. The Box plots represent in the horizontal bar the mean value for mRNA in each group. The whiskers represent the maximum and minimum values of the data. Black dots represent outliers. **P*<0.05; $^{\#}$*P*=0.058.

The mRNA expression of *ABCC4* genes was particularly differentiated in the rs1678405, rs1751031 and rs869951 tagSNPs. The *ABCC4* levels were progressively lower in rs1678405TC and CC genotypes in malignant mucosa (-2.26±1.47 and -3.39±0.92, respectively, vs -1.43±1.18 with the TT genotype, *P*=0.049), as noticed in Table 5. A 50% decrease in mRNA expression was noticed with the rs1751031AG genotype in normal-appearing tissues (-3.83±1.44 vs -2.77±1.30, *P*=0.019). Furthermore, a nearly 9-fold overexpression was observed with the rs869951GC genotype (0.74±1.33 vs -2.39±0.93 for the GG genotype, *P*=0.001). Furthermore, a higher expression of *ABCC4* was observed with the rs1751051AA genotype in normal-appearing tissues in contrast with genotypes carrying the T allele, although not statistically significant (-2.28±1.28 vs -3.18±1.41, respectively, *P*=0.118). A trend for increased expression was reported in the presence of rs3742106C allele (-3.38±1.27 vs -2.79±1.45 for the AA genotype, *P*=0.113 in normal-appearance mucosa and -1.47±1.33 vs -2.21±1.24 in CRC tissues, *P*=0.096).

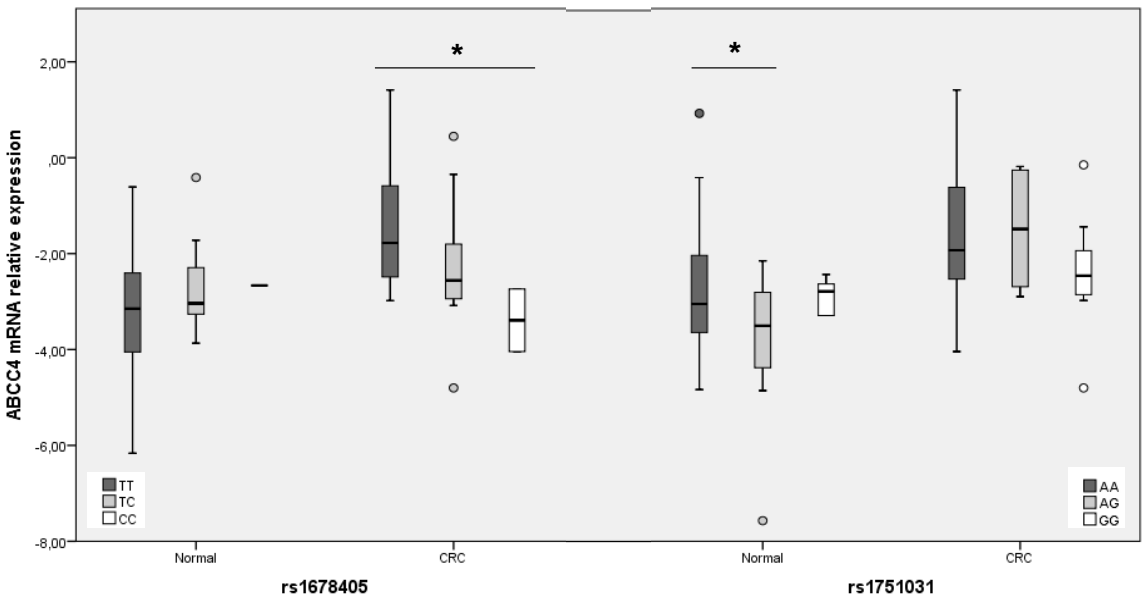


Figure 2. Relative levels of *ABCC4* mRNA (-ΔCT) considering the genotypes for the rs1678405T>C (left side) and rs1751031A>G (right side) polymorphisms. The Box plots represent in the horizontal bar the mean value for mRNA in each group. The whiskers represent the maximum and minimum values of the data. Black dots represent outliers. **P*<0.05.

DISCUSSION

Colorectal cancer develops through a multistep process, known as the adenoma-carcinoma sequence, as a result of the cumulative effects of acquired molecular events due to genomic instability and the influence of a large number of low-penetrance genetic variants each exerting a modest effect on CRC risk [1,23,24].

Deregulation of the COX-2/PGE_2 pathway is an early event in colorectal carcinogenesis leading to the stimulation of a plethora of oncogenic pathways with repercussion on most if not all hallmarks of cancer [9,11].

In this functional study we assessed the influence of a set of polymorphisms formerly implicated in colorectal carcinogenesis [18,19] on *COX-2*, *HPGD*, *SLCO2A1* or *ABCC4* genes' expression in colonic tissues.

Surprisingly, we observed a stable mRNA expression of *HPGD* from normal-appearing to malignant mucosa in contrast with previous works. Yan and colleagues [25] observed a high expression of *HPGD* in normal mucosa but that for most of colon cancer samples the *HPGD* mRNA expression was at undetectable levels. Considering that we only included tissues with measurable

gene expression, we might have an enrichment towards higher mRNA levels in CRC samples, potentially explaining the disparities observed with earlier reports. Holla and colleagues [17] observed that *SLCO2A1* mRNA expression was significantly lower in fresh-frozen human colorectal tumor specimens than that noticed in matched normal mucosa. We failed to corroborate this finding. In fact, we observed a two-fold overexpression in CRC tissues. Nevertheless, and similarly to our results, Lejeune and colleagues [26] noticed that PGT expression was up-regulated in colonic epithelial cells during inflammation (ulcerative colitis) leading to a high-output of PGE_2 extracellularly by vectorial transport through the epithelium towards lumen.

Despite these inconsistencies, a significant variation was observed in gene expression across all genes (above seven-fold), suggesting the involvement of genetic determinants.

The regulation of mRNA levels by different alleles appears to be dependent on the histological context. During colorectal carcinogenesis different pathways are activated overtime due to acquired mutations or environmental exposure resulting on the release of transcription factors (TF) that can bind to the regulatory regions containing the genetic variations, thus leading to an altered gene expression. As an example, mutations in adenomatous polyposis coli (APC) gene is associated with the earliest stages of colorectal carcinogenesis and shown to increase COX-2 expression through C/EBP-β [27]. If a specific *COX-2* polymorphisms is located within the recognition-binding site for that TF a differential expression between alleles will be noticed once that pathway is activated.

The rs689466G>A polymorphism in *COX-2* gene was previously associated with an increased risk for the development of colorectal cancer and adenomas [18,19]. Its role on cancer is controversial. Initial studies in Asian populations identified the GG genotype as risk marker for the occurrence of gastrointestinal cancers and a higher *COX-2* mRNA expression was observed in esophageal tumors [28-30]. In CRC cell lines the opposing GG genotype let to an increase in *COX-2* transcriptional activity [31]. This biological repercussion was further supported in the present study, where a 7-fold overexpression was reported in CRC specimens.

Four genetic variants in *HPGD* gene had an allele-specific mRNA gene expression (rs1863642G>T, rs1346271G>C, rs1426945G>A and rs2555639T>C) that

provided the biological reasoning underlying the epidemiological observations, with the exception of rs1863642G>T, although for some, the difference was not statistically significant probably reflecting limited statistical power. This genetic variant, located in an intronic region with no predicted impact on protein expression or function, was associated with a decreased in *HPGD* expression and antagonically a protection was reported for the occurrence of colorectal adenomas in the presence of T allele. Future studies are needed for a deeper comprehension on the influence of this tagSNP or other represented in the LD block on colorectal carcinogenesis.

Looking at the *SLCO2A1* gene, the AA genotype of rs6439448C>A polymorphism presented a trend for higher mRNA expression in neoplastic sample. Following the traditional view on PGT, increased levels of *SLCO2A1* will increase the cellular intake of PGE_2 and subsequent inactivation by 15-PGDH, thus explaining the lower risk observed for colorectal tumors. Although this tagSNPs has no predictable function repercussion (SNPinfo database) it tags two SNP: the rs2370512T>C located in the 3'UTR that could influence mRNA expression and stability and the rs34550074G>A at the codon 396 coding for two difference aminoacids.

Five out of the nine tagSNPs that altered the susceptibility for the development and recurrence of colorectal tumors influenced the *ABCC4* mRNA expression levels corroborating their relevance in colorectal carcinogenesis. The rs1751031AG genotype was associated with a lower expression in contrast with the AA homozygous genotype. That under-expression is expected to hamper the efflux-dominated flow decreasing the levels of PGE_2 in the extracellular milieu and the activation of oncogenic pathways thus explaining the lower risk observed.

Gene expression studies from FFPE represent an unlimited source for translational cancer research. Unfortunately, this type of analysis may be problematic considering the potentially low quality of the RNA extracted from FFPE [32,33]. To overcome this limitation we selected amplicons under 100bp proven to obtain accurate and specific gene expression in Real-Time analysis [32]. We also carried-out the reverse transcriptase step with random primers, considering that up to 50% of RNA may not contain the poly-A tail intact [33].

Furthermore, we also observed a stable expression of the *KRT20* reference gene across samples.

In conclusion, the present functional study reports the involvement of genetic variants on *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* genes' expression providing a biological reasoning underlying the epidemiological data. This further reinforces the important role that the genetic background has on the development of colorectal tumors. The definition of higher-risk populations might be a useful tool for targeted screening and surveillance. Furthermore, Chan and colleagues [34] reported that the protective role of aspirin in colorectal carcinogenesis is only noticeable in tumors overexpressing COX-2. So, the identification of polymorphisms that influence the genes' levels might be particularly relevant for selecting subjects for chemopreventive strategies.

REFERENCES

[1] Balmain A, Gray J, Ponder B. The genetics and genomics of cancer. Nat Genet 2003;33(Suppl): 238–44

[2] Morley M, Molony CM, Weber TM, et al. Genetic analysis of genome-wide variation in human gene expression. Nature 2004;430:743-7.

[3] Flossmann E, Rothwell PM; British Doctors Aspirin Trial and the UK-TIA Aspirin Trial. Effect of aspirin on long-term risk of colorectal cancer: consistence form randomized and observational. Lancet 2007;369(9573):1603-13

[4] Cole BF, Logan RF, Habali S, et al. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. J Natl Cancer Inst 2009;101(4):256-66.

[5] Sandler RS, Halabi S, Baron JA, et al. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. N Engl J Med 2003;348:883–90.

[6] Chulada PC, Thompson MB, Mahler JF, et al. Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. Cancer Res 2000;4705-8.

[7] Wang D, Wang H, Shi Q, et al. Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. Cancer Cell. 2004;6:285–95.

[8] Vane JR, Blotting RM. Mechanism of action of nonsteroidal anti-inflammatory drugs. Am J Med 1998;104(3A):2S-8S;

[9] Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, Dubois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107(4):1183-8.

[10] Reid G, Wielinga P, Zelcer N, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci USA 2003;100:9244–9.

[11] Wang D, Mann JR, DuBois RN. The role of prostaglandins and other eicosanoids in the gastrointestinal tract. Gastroenterology 2005;128(5):1445-61.

[12] Kawamori T, Uchiya N, Sugimura T, et al. Enhancement of colon carcinogenesis by prostaglandin E2 administration. Carcinogenesis 2003;24:985-90.

[13] Nomura T, Lu R, Pucci MK, et al. The two-step model of prostaglandin signal termination: *In vitro* reconstitution with the prostaglandin transporter and prostaglandin 15-dehydrogenase. Mol Pharmacol 2004;65:973-8.

[14] Backlund MG, Mann JR, Holla VR, et al. 15-Hydroxyprostaglandin Dehydrogenase is Down-regulated in Colorectal Cancer. J Biol Chem 2005;280(5):3217-23.

[15] Myung SJ, Rerko RM, Yan M, Buchanan FG, Tai HH, Musiek ES, Milne GL, Katkuri S, Dubois RN. 15-Hydroxyprostaglandin dehydrogenase is an in vivo suppressor of colon tumorigenesis. PNAS 2006;103(32):12098-102.

[16] Yan M, Myung SJ, Fink SP, et al. 15-Hydroxyprostaglandin dehydrogenase inactivation as a mechanism of resistance to Celecoxib chemoprevention of cólon tumors. PNAS 2009;106(23):9409-13.

[17] Holla VR, Backlund MG, Yang P, et al. Regulation of Prostaglandin Transporters in Colorectal Neoplasia. Cancer Prev Res 2008;1(2):93-9

[18] Pereira C, Queirós S, Galaghar A, et al. Genetic variability in key genes in prosyaglandin E2 pathway (COX-2, HPGD, ABCC4 and SLCO2A1) and their involvement in colorectal cancer development. PLoS One 2014;9(4):e92000. doi: 10.1371/journal.pone.0092000. eCollection

[19] Pereira C, Queirós S, Galaghar A, et al. Polymorphisms in prostaglandin E2 (PGE_2) pathway genes alter the risk for colorectal adenoma recurrence after polypectomy: a chance for individualized surveillance? (Submitted for publication)

[20] Thompson CL, Fink SP, Lutterbaugh JD, et al. Genetic variation in 15-Hydroxyprostglandin Dehydrogenase and colon cancer susceptibility. PLoS one 2013:8(5):e64122

[21] Myung SJ, Rerko RM, Yan M, et al. 15-Hydroxyprostgalandin dehydrogenase is an in vivo suppressor of colon tumorigenesis. Proc Natl Acad Sci USA 2006;103:12098-102.

[22] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantification PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402-8.

[23] Fearon Er, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990;61(5):759-67.

[24] Vogelstein B, Kinzler KW: The multistep nature of cancer. Trends Genet 1993;9(4):138-41.

[25] Yan M, Myung SJ, Fink SP, et al. 15-Hydroxyprostaglandin dehydrogenase inactivation as a mechanism of resistance to Celecoxib chemoprevention of cólon tumors. PNAS 2009;106(23):9409-13.

[26] Lejeune M, Leung P, Beck PL, et al. Role of EP4 receptor and prostaglandin transporter in prostaglandin E_2-induced alteration in colonic epithelial barrier integrity. Am J Physiol Gastrointest Liver Physiol 2010;299:G1097–G05.

[27] Eisinger AL, Nadauld LD, Shelton DN, et al. The Adenomatous Polyposis Coli Tumor Suppressor Gene Regulates Expression of Cyclooxygenase-2 by a

Mechanism That Involves Retinoic Acid*. J Biol Chem 2006;281:20474-82.

[28] Liu F, Pan K, Zhang X, et al. Genetic variants in cyclooxygenase-2: Expression and risk of gastric cancer and its precursors in a Chinese population. Gastroenterology 2006;130:1975-84.

[29] Zhang X, Miao X, Tan W, et al. Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer. Gastroenterology 2005;129:565-76.

[30] Tan W, Wu J, Zhang X, et al. Associations of functional polymorphisms in cyclooxygenase-2 and platelet 12-lipoxigenase with risk of occurrence and advanced disease status of colorectal cancer. Carcinogenesis 2007;28:1197-201.

[31] Pereira C, Sousa H, Silva J, et al. The -1195A>G allele increases the transcriptional activity of cyclooxygenase-2 gene (COX-2) in colon cancer cell lines. Mol Carcinog 2014;53 Suppl1:E92-5.

[32] Farragher SM, Tanney A, Kennedy RD, et al. RNA expression analysis from formalin fixed paraffin embedded tissues. Histochem Cell Biol 2008;130:435-45.

[33] Masuda N, Ohnishi T, Kawamoto S, et al. Analysis of chemical modifications of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids res 1999;27:4436-43.

[34] Chan AT, Ogino S,Fuchs CS. Aspirin and the risk of Colorectal cancer in relation to the expression of COX-2. N Engl J Med 2007;356:2131-42.

Table S1. Description of genetic polymorphisms previously implicated in Colorectal carcinogenesis

Gene / SNP	Nucleotide substitution	Location	Previous epidemiological association	
			CRC	Adenoma
COX-2				
rs689466	A>G	5'UTR	Risk (AAvsGG)	Risk (AA/AGvsGG)
HPGD				
rs12500316	C>T	Intron		Protection (GGvsGT/TT)
rs1863642	G>T	Intron		Protection (GGvsGT/TT)
rs1346271	G>C	5'UTR	Protection (GGvsGC)	Protection (GGvsGC/CC)
rs1426945	G>A	5'UTR	Protection (GGvsAA)	-
rs2555639	T>C	5'UTR		Risk (TT/TCvsCC)
SLCO2A1				
rs6439448*	C>A	Intron	Protection (CCvsCA)	Protection (CCvsCA/AA)
rs1131598	A>G	3'UTR		Protection (AAvsAG/GG)
rs7616492	G/A	Intron	Risk (GGvsAA)	Higher interval[#] (GGvsGA/AA)
rs9820625	A>C	Intron		Risk (AA/ACvsCC)
rs7340717	G>T	Intron		Lower interval (GG/GTvsTT)
ABCC4				
rs9524821	G>A	Intron		Risk (GG/GAvsAA)
rs1751051	T>A	Intron	Risk (TTvsAA)	Risk (TT/TAvsAA)
rs1678405	T>C	Intron		Protection (TTvsTC/CC)
rs1751031	A>G	Intron	Protection AAvsAG	Lower time -
rs1678396	T>C	Intron		Protection (HR) TTvsTC/CC
rs2274403	A>G	Intron		Protection (HR) AAvsAG/GG
rs3742106	A<C	3'UTR		Risk (HR) AAvsAC/CC
rs6492763	T<C	Intron		Protection (HR) TTvsTC/CC
rs869951	G<C	5'UTR		Lower time GG/GCvsCC

* Tags the rs2370512T>C in 3'UTR and rs34550074G>A at codon 396 that codes for diffferent aminoacids; [#] Time interval until the recurrence of colorectal adenomas.
HR, hazard ratio for recurrence of Colorectal adenomas

CHAPTER VII: GLOBAL DISCUSSION & MAIN CONCLUSIONS

An increasingly large body of evidence highlight the predominant role of COX-2/PGE_2 pathway in promoting cancer progression through multiple signaling pathways, as reviewe by several authors [1,2]. Briefly, once synthesized by COX-2 the PGs are transported into the extracelular microenvironment by the specific MRP4 transporter, where PGE_2 binds to G-protein coupled receptors stimulating downstream signals such as cAMP or PI3K [3]. After PGE_2 is returned back to the cytoplasm by the influx PGT transporter it is enzymatically catabolized by 15-PGDH [4,5]. Deregulation of this pathway through an overexpression of COX-2/MRP4 and inversevely a downregulation of PGT/15-PGDH expression leads to the accumulation of PGE_2 in microenvironment [7-12].

In this thesis, we hypothesized that the genetic background might contribute to the deregulation of PGE_2 pathway, with potential repercursion on the susceptibility for colorectal tumors development. Furthermore, a deeper understanding on colorectal carcinogenesis through the identification and characterization of genetic biomarkers will allow the characterization of individuals at higher risk for CRC that in the foreseeable future could offer a clinical reasoning.

Early screening and follow-up of individuals previously diagnosed with colorectal adenomas is the cornerstone of CRC prevention [13,14]. Nevertheless, the adherence rates in countries with implemented population-based CRC screening guidelines, are far from the desirable for a successful decrease in CRC burden [15]. Complementary, the regular use of NSAIDs has been consistently effective in the prevention of colorectal tumors development and recurrence [16-19]. Nevertheless, concerns over its safety has haulted their applicability as chemopreventive agents in cancer at least in average-risk population [20].

So, the challenge falls in the identification of biomarkers that could target higher-risk populations for colorectal screening and/or chemopreventive strategies.

To test our hypothesis, two observational studies were designed to address the involvement of genetic variability on *COX-2, HPGD, SLCO2A1* and *ABCC4* genes, coding for the COX-2, 15-PGDH, PGT and MRP4 proteins, respectively, on the susceptibility not only for CRC (Chapter IV) but also on the development of early stage colorectal tumors (Chapter V).

Cohort studies provide the strongest scientific evidence among observational studies by creating a time framework that establishes the causality between the

variable of exposure and outcome, by following individuals over time, thus minimizing the influence of selection and recall bias [21]. Neverthless, the long latency period of CRC (10-15 years) and the relatively high number of subjects needed to be recruited (nearly 3400 individuals to detect a RR of 1.70 with 95% confidence interval and 80% power; MAF=15% and CRC lifetime risk =4%) that would translate in an overly expensive and time consuming approach hindered our capacity to follow this study design during the time frame of this project.

Hence, we implemented a case-control study gathering patients diagnosed with CRC at a reference oncological centre for the entire North of Portugal (Chapter IV). Considering the time interval between cancer diagnosis and the beginning of this project we cannot exclude survival bias, though we did not observed any difference in genotypes distribution among clinicopathological variables that correlate with survival rate, namely stage of disease. Our control group was made up of unscreened blood donors from the same institute. Eighty-five percent of these participants were asymptomatic and still blood donors five to seven years after recruitment. Most of the remainder subjects quit being donors due to age criteria and there were no record of CRC. Taken together it is highly unlikely that crossover had occur. The same control group was used in the case-control study involving patients with personal history of colorectal adenomas (Chapter V). This might not be the most suitable control subjects, considering the high prevalence of adenomatous polyps and absence of symptomatology normaly associated with these lesions [22,23]. Neverthless, and as discussed in Chapter V, if selection bias was evident the directionality of our results is expected to remain but the magnitude of associations would tend to be more noticeable. Furthermore and although we did not matched controls to cases in the design phase we minimized the effect of potential confounding variables in the statistical analysis either through data stratification or multivariate analysis.

In the inability to include independent replication test sets to address the generalisability of the results reported here, we assessed the predictive accuracy by resampling the original data following the bootstrap method [24]. Most of our association were proven to be rather stabe an robust, as mentioned in the individual studies.

The release of the first human genome draft highlighted the relevance of common genetic variations along side the development of high throughput genotyping platforms have shifted the way diseases are studied from evaluating a few promising SNPs in candidate genes to hundreds or even thousands.

The false positive report probability (FPRP) was used to correct for multiple testing and address possible false positive findings [25]. This method calculates the probability of no association given a statistical significant finding. Unlike more stringent approaches, like Bonferroni correction that focus solely on the number of test performed, the determinants of FPRP magnitude include: (1) prior probability of a true association; (2) observed *P* value and (3) statistical power to detect the *odds ratio* of the alternative hypothesis at the given *P* value. We set the FPRP noteworthiness value at 0.5 recommended for small initial studies [25]. The prior probability cutoff depends on the availability of previous epidemiological and/or functional data, also as biological plausibility, thus explaining the different values assumed in Chapter V (Table 2). The associations observed on the recurrence of adenomas were more susceptible to represent false positive findings, that could reflect the lower statistical power.

In this project, we took advantage of the extensive linkage desiquilibrium (LD) regions reported across the human genome, based on the assumption that a complete set of sequence variants within these blocks bear redundant information that can be significantly reduced to a subset of tagging markers (tagSNPs) [26]. This set of tagSNPs can be use to capture the vast majority of SNP variation in a region, thereby reducing significantly the genotyping costs and sample amounts [26]. We retrieved our panel of tagSNPs from the CEU population of the International HapMap Project, that is represented by Utah residents with northern and western European ancestry [27]. The tagSNPs transferability as been addressed in several studies [28-30]. Gu S and colleagues [30], observed a high tagSNPs portability among European populations that captured an average of 95% of common variants. Nevertheless, considerable heterogeneity in LD pattern was noticed that could explain the discrepancies reported between the original and subsequent replication studies [30]. Nearly 40% of the tagSNPs recovered from the CEU population were included in this study.

One obstacle that we were faced when trying to increase our sample set from the preliminary study descrived in Chaper IB and gathered the subjects with previous history of colorectal adenomas in Chapter V, was the inability to obtain blood samples. This was overcomed by isolating DNA from archived formalin-fixed, paraffin-embedded (FFPE) tissues. Although representing a valuable an extensive source in biomedical research its use has been rather neglected considering the (1) expected chemical modifications and nucleic acids degradation; and (2) occurrence of somatic changes during tumor development [31,32]. Horn H and colleagues [33] reported a perfect concordance rate between genotypes from germline DNA and FFPE speciments when using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with sequenom iPLEX technology and high genotyping detection rates (over 90%). This was further corroborated by others [34] and also observed when using TaqMan genotyping assays [35,36]. We internally validated the use of FFPE tissues by genotyping 20 somatic and paired germline DNAs from patients diagnosed with CRC. A concordance rate of 100% was noticed. The genotype call rates and concordance rates between replicates were high in both observational studies (97 and 99% in the studies reported in Chapter IV and V, respectively), although the call rate was lower in patients with adenomas (94%) probably due to the low amounts of DNA extracted from small polyps (<2mm).

After these methodological considerations we will focus on providing an overview of main findings integrating this PhD thesis. In the individual studies each worthmentioning SNP is explored.

Common diseases such as cancer and cardiovascular diseases are believed to develop through a combination of genetic and environmental factors [37-39]. Because genes often act in group to perform a specific biological function or cellular process, we decided to investigate the contribution of common genetic variants in the four key genes regulating PGE_2 levels in the extracellular milleu (*COX-2/HPGD/SLCO2A1/ABCC4*) on colorectal carcinogenesis. Furthermore, understanding how these genetic markers interact with each other and with the environment might allow the definition of more accurate risk models that could contribute to decrease CRC burden through individualized prevention.

To the best of our knowledge the studies described in Chapters IV and V are the first using this integrative approach. Previous reports focused their attention mainly on the PGE_2 synthesis axis and more recently in 15-PGDH or other pathways in arachidonic acid metabolism [40-50].

Most of the polymorphisms identified here as biomarkers appear to have a tumor stage-dependent behavior. In other words, different SNPs significantly contribute to CRC development in diferent phases of colorectal carcinogenesis. Although immutables, most SNPs are located in noncoding region of genes, namely in portions regulating genes' transcription and mRNA stability [51-54]. The differential expression of nuclear proteins or microRNA from adenomas to carcinomas might modulate these SNPs behavior during colorectal oncogenesis [55]. As an example, the rs1131598A>G polymorphism is found in the 3'UTR of *SLCO2A1* gene and bioinformatically (SNPinfo software) the presence of G allele eliminates the binding site for microRNA-136. Normally, microRNAs down-regulate the expression of target genes either through mRNA degradation or blocking of protein translation, although no information is available regarding the expression of this microRNA in colorectal tumors [56]. If and hypothesizing that miR-136 is preferential expressed in colorectal adenomas this would mean that the rs1131598G allele would disrupt the binding of this microRNA to 3'UTR leading to a higher expression of PGT thus explain the lower risk observed for colorectal adenomas. The same would not be observed in CRC if in fact no expression of miR-136 was observed. The rs689466A>G in *COX-2* gene was one of four SNPs that impacted colorectal carcinogenesis in both early and later stages of tumor development. An increase on the risk for colorectal adenoma and cancer was reported, that was even more noticeable in men and ever-smokers. This finding supports the observations from the preliminary study described in Chapter IB [57]. Furthermore, the presence of G allele was associated with a higher *COX-2* transcriptional activity in two CRC cell lines, as reported in Chapter III and to a 7-fold *COX-2* mRNA overexpression, in contrast with the AA genotype, as described in Chapter VI. With this study we provided for the first time a biological reasoning underlying the epidemiological data in CRC, although further *in vitro* studies are warranted to unravel the interaction between the rs689466A>G SNP and smoke consumption that could uncover new molecular targets for CRC prevention.

Stage-specific genetic profiles were observed in the gene-gene interaction analysis that significantly improved the magnitude of the impact of the genetic background, lending further support to the common variant common disease hypothesis [39]. In adenomas the four-factor interaction model including polymorphisms in *HPGD* and *ABCC4* genes increased by 13-fold the genetic predisposition. Furthermore, a nearly 80% accuracy for predicting the development of adenomatous polyps was observed. The multilocus analysis implemented in this project may help define genetic signatures for the development of colorectal tumors with potential repercussion on CRC prevention. If corroborated by further studies, this approach might provide insightful clues on how these genes regulate each other.

The current guidelines for surveillance post-polypectomy are solely based on the endoscopic findings at baseline colonoscopy [58]. In Chapter V we also observed that certain genetic variations could influence not only the interval time to recurrence of colorectal adenomatous polyps but also the crude risk for recurrence at 36, 60 and 120 months. As an example, nearly half of individuals carrying the rs9524821AA genotype in the low-risk group developed metachronous lesions by 60 months in contrast with the 16% reported for G allele carriers. This appears to suggest that subjects with rs9524821AA genotype might benefit from a shorter interval between surveillance colonoscopy.

The involvement of genetic variants in *COX-2*, *HPGD*, *SLCO2A1* and *ABCC4* genes in colorectal carcinogenesis was further supported, at least for some, by the findings from the functional study evaluating the repercussion of SNPs in genes' mRNA expression, reported in Chapter VI.

In conclusion, this study provided new insights into colorectal carcinogenesis through the identification of new genetic biomarkers in COX-2/PGE_2 pathway for different stages of colorectal cancer development. We further corroborated the influence of rs689466A>G (-1195A>G) *COX-2* polymorphism in colorectal carcinogenesis and provided a biological explanation underlying the epidemiological data. For the first time, we defined stage-specific multilocus models reflecting the cumulative effect of SNPs in *COX-2, HPGD, SLCO2A1* and *ABCC4* genes. Furthermore, an influence on the time to recurrence and recurrence rates of adenomas at 36, 60 and 120 months was also noticed for

polymorphisms in *SLCO2A1* and *ABCC4* genes. Taken toghether, the genetic variability in key genes of COX-2/PGE_2 pathway might help select individuals at higher risk for colorectal tumors that could benefit from targeted screening/surveillance post-polypectomy or even be included in chemopreventive strategies, specially those overexpressing COX-2 or 15-PGDH enzymes. The preventive effects of regular use of aspirin were specifically reported in patients overexpressing COX-2 or 15-PGDH [59,60].

REFERENCES

[1] Wang D, Mann JR, Dubois RN. The role of prostaglandin and other eicosanoids in gastrointestinal tract. Gastroenterology 2005;128:1445-61.

[2] Dixon DA, Bianco FF, Bruno A, et al. Mechanistic aspects of COX-2 expression in Colorectal neoplasia. Recent Results cancer Res 2013;191:7-37.

[3] Reid G, Weilinga P, Zelcer N, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflamatory drugs. Proc Natl Acad Sci 2003;100(16):9244-9.

[4] Schuster VI. Prostaglandin transport. Prostaglandins Other Lipid Mediat 2002;68-69:633-47.

[5] Tai HH, Ensor CM, Tong M, et al. Prostaglandin catabolizing enzymes. Prostaglandins Other Lipid Mediat 2002;68-69:483-93.

[6] Holla VR, Backlund MG, Yang P, et al. Regulation of prostaglandin transporters on colorectal neoplasia. Cancer Prev Res 2008;1(2):93-9.

[7] Eberhart CE, Coffey RJ, Radhika A, et al. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenoma and adenocarcinoma. Gastroenterology 1994;107:1183-88.

[8] Gupta RA and Dubois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2 . Nat Rev Cancer 2001;1:11-21.

[9] Marnett LJ and Dubois. COX-2: a Target for colon cancer prevention. Annu Rev Pharmacol toxicol 2002;42:910-4.

[10] Backlund MG, Mann JR, Holla VR, et al. 15-hydroxyprostglandin dehydrogenase is down-regulated in colorectal cancer. J Biol Chem 2005;280:3217-23.

[11] Myung SJ, Rerko RM, Yan M, et al. 15-hydroxyprostaglandin dehydrogenase is an in vivo supressor of cólon tumorigenesis. Proct Natl Acad Sci USA 2006;103:12098-102.

[12] Yan M, Rerko RM, Platzer P, et al.15-hydroxyprostaglandin dehydrogenase, a COX-2 oncogene antagonista, is a tgf-beta-induced suppressor of human gastrointestinal cancers. Proc Natl Acad Sci USA 2004;101:17468-73.

[13] Atkin WS, Edwards R, Kralj-Hans, et al. Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a muiticentre randomized controlled trial. Lancet 2010;375:1624-33.

[14] Zauber AG, Winawer SJ, O'Brien MJ, et al. Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. N Engl J Med 2012;366:687-96.

[15] Pox CP, Altenhofen L, Brenner H, et al. Efficacy of a nationwide screening colonoscopy program for colorectal cancer. Gastroenterology 2012;142(7):1460-7.

[16] Flossmann E, Rothwell PM; British Doctors Aspirin Trial and the UK-TIA Aspirin Trial. Effect of aspirin on long-term risk of colorectal cancer: consistence form randomized and observational. Lancet 2007;369(9573):1603-13.

[17] Rothwell PM, Wilson M, Elwin CE, et al. Lond-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomized trials. Lancet 2010;376(9654):1741-50.

[18] Cole BF, Logan RF, Habali S, et al. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of te randomized trials. J Natl Cancer Inst 2009;101(4):256-66.

[19] Gao F, Liao C, Liu L, et al. The effect of aspirin in the recurrence of Colorectal adenomas: a meta-analysis of randomized controlled trials. Colorectal Dis 2009;11(9):893-901.

[20] Rodríguez LA and Tolosa LB. Risk of upper gastrointestinal complications among users of traditional NSAIDs and COXIBs in the general population. Gastroenterology 2009;132(2):4498-506.

[21] Song JW and Chung KC. Observational studies: Cohort and Case-Control Studies. Plast Reconstr Surg 2010;126(6):2234-42.

[22] Lieberman DA, Weiss DG, Bond JH, et al. Use of colonoscopy to screen asymptomatic adults for colorectal cancer. N Engl J Med 2000;343(3):162-8.

[23] Gupta N, Bansal A, Rao D, et al. Prevalence of advanced histological features in diminutive and small colon polyps . Gastrointest Endosc 2012;75:1022-30.

[24] Molinaro AM, Simon R, Pfeiffer RM. Prediction error estimation: a comparison of resampling methods. Bioinformatics 2005;21(15):3301-7.

[25] Wacholder S, Chanock S, Garcia-Closas M, et al. Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. J Natl Cancer Inst. 2004 Mar 17;96(6):434-42.

[26] Johnson GC, Esposito L, Barratt BJ, et al. Haplotype tagging for the identification of common disease genes. Nat Genet 2001;29:233–7.

[27] The International HapMap Consortium. The International HapMap Project. Nature 2003;426:789-96.

[28] Mueller JC, Lohmussaar E, Reedik Magi, et al. Linkage Disequilibrium Patterns and tagSNPs Transferability among European Populations. Am J Hum Genet 2005;76:387-98.

[29] E.M. Smith, X. Wang, J. Littrell, et al. Comparison of linkage disequilibrium patterns between the HapMap CEPH samples and a family-based cohort of Northern European descent. Genomics 2006;88(4):407-14.

[30] Gu S, Pakstis AJ, Li H, et al. Significant variation in haplotype block structure but conservative in tagSNPs pattern among global populations. Eur J Hum Genet 2007;15(3):302-12.

[31] Farragher SM, Tanney A, Kennedy RD, et al. RNA expression analysis from formalin fixed paraffin embedded tissues. Histochem Cell Biol 2008;130:435-45.

[32] Masuda N, Ohnishi T, Kawamoto S, et al. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 1999;27:4436-43.

[33] Horn H, Pott C, Kalla J, et al. A multiplex MALDI-TOF MS approach facilitates genotyping of DNA from formalin-fixed paraffin-embedded tumour specimens. Pharmacogenet Genomics 2010;20(10):598-604.

[34] Li AL, Song YX, Wang ZN, et al. Polymorphisms and a haplotype in heparanase gene associations with the progression and prognosis of gastric cancer in a northern Chinese population. PLoS One 2012;7(1):e30277. doi: 10.1371/journal.pone.0030277.

[35] Jaremko M, Justenhoven C, Abraham BK, et al. MALDI-TOF MS and TaqMan assisted SNP genotyping of DNA isolated from formalin-fixed and paraffin-embedded tissues (FFPET). Hum Mutat 2005;25(3):232-8.

[36] Hagleitner MM, Coenen MJ, Jeuken JW, et al. Taqman genotyping assays can be used on decalcified and paraffin-embedded tissue from patients with osteosarcoma. Pediatr Blood Cancer 2011;56(1):35-8.

[37] Wright AF, Carothers AD, Campbell H. Gene–environment interactions—the BioBank UK study. Pharmacogenomics J 2002;2(2):75-82.

[38] Nickels S, Truong T, Hein R, et al. Evidence of gene-environment interactions between common breast cancer susceptibility loci and established environmental risk factors. PLoS Genet 2013;9(3): e1003284. doi: 10.1371/journal.pgen.1003284.

[39] Hemminki K, Forsti A, Bermejo JL. The 'Common Disease-Common Variant' Hypothesis and Familial Risks. PLoS ONE 2008; 3(6): e2504. doi:10.1371/journal.pone.0002504

[40] Peng Q, Yang S, Lao X, et al. Meta-Analysis of the Association between Polymorphisms and Risk of Colorectal cancer Based on Case-Control Studies. PLoS One 2014;9(4):e94790. doi: 10.1371/journal.pone.0094790.

[41] Andersen V, Holst R, Kopp TI, et al. Interactions between diet, lifestyle and IL10, IL1B, and PTGS2/COX-2 gene polymorphisms in relation to risk of colorectal cancer in a prospective Danish case-cohort study. PLoS One 2013; 23;8(10):e78366. doi: 10.1371/journal.pone.0078366.

[42] Li S, Zhao X, Wu Z , et al. Polymorphisms in arachidonic acid metabolism-related genes and the risk and prognosis of colorectal cancer. Fam Cancer 2013;12(4):755-65

[43] Thompson CL, Fink SP, Lutterbaugh JD, et al. Genetic variation in 15-hydroxyprostaglandin dehydrogenase and colon cancer susceptibility. PLoS One 2013;22;8(5):e64122. doi: 10.1371/journal.pone.0064122.

[44] Poole EM, Hsu L, Xiao L, et al. Genetic variation in prostaglandin E2 synthesis and signaling, prostaglandin dehydrogenase, and the risk of colorectal adenoma. Cancer Epidemiol Biomarkers Prev 2010;19(2):547-57.

[45] Barry EL, Sansbury LB, Grau MV, et al. Cyclooxygenase-2 polymorphisms, aspirin treatment, and risk for colorectal adenoma recurrence--data from a randomized clinical trial. Cancer Epidemiol Biomarkers Prev 2009;18(10):2726-33.

[46] Siezen CL, van Leeuwen AI, Kram NR, et al. Colorectal adenoma risk is modified by the interplay between polymorphisms in arachidonic acid pathway genes and fish consumption. Carcinogenesis 2005;26(2):449-57.

[47] Poole EM, Bigler J, Whitton J, et al. Prostacyclin synthase and arachidonate 5-lipoxygenase polymorphisms and risk of colorectal polyps. Cancer Epidemiol Biomarkers Prev 2006;15(3):502-8.

[48] Edwards TL, Shrubsole MJ, Qiuyin C, et al. A Study of Prostaglandin Pathway genes and Interactions with Current Nonsteroidal Anti-inflammatory Drug Use in Colorectal Adenoma. Cancer Prev Res 2012;5:855-63.

[49] Hoeft B, Linseisen J, Beckmann L, et al. Polymorphisms in fatty acid metabolism-related genes are associated with colorectal cancer risk. Carcinogenesis 2010;31(3):466-72.

[50] Frank B, Hoeft B, Hoffmeister M, et al. Association of hydroxyprostaglandin dehydrogenase 15-(NAD) (*HPGD*) variants and colorectal cancer risk. Carcinogenesis 2010;32(2):190-6.

[51] Guo Y and Jamison DC. Te distribution of SNPs in human gene regulatory regions. BMC Genomics 2005;6:140.

[52] Zhao D, Xu D, Zhang X, et al. Interaction of cyclooxygenase-2 variants and smoking in pancreatic cancer: a possible role of nucleophosmin. Gastroenterology 2009;136(5):1659-68.

[53] Zhang X, Miao X, Tan W, et al. Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer. Gastroenterology 2005;129(2):565-76.

[54] Moore AE, Young LE, Dixon DA. A common single-nucleotide polymorphism in cyclooxygenase-2 disrupts microRNA-mediated regulation. Oncogene 2012;31(12):1592-8.

[55] Pesson M, Volant A, Uguen A, et al. A Gene Expression and Pre-mRNA Splicing Signature That Marks te Adenoma-Adenocarcinoma Progression in Colorectal Cancer. PLoS One 2013;9(2). e87761. doi:10.1371/journal.pone.0087761

[56] Fabian MR, Sonenberg N, Filipowitz W. Regulation of mRNA translation and stability by microRNA. Annual Review of Biochemistry 2010;79:351-79.

[57] Pereira C, Pimentel-Nunes P, Brandão C, et al. COX-2 polymorphism and colorectal cancer risk: a strategy for chemoprevention. Eur J Gastroenterol Hepatol 2010;22(5):607-13.

[58] Hassan C, Quintero E, Dumonceau JM, et al. Post-polypectomy colonoscopy surveillance: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. Endoscopy 2013;45:842:51.

[59].Chan AT, Oino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. N Engl J Med 2007;356:2131-42.

[60] Fink SP, Yamauchi M, Nishihara R, et al. Aspirin and the risk of colorectal cancer in relation to the expression of 15-Hydroxyprostaglandin dehydrogenase (HPGD). Sci Transl Med;6(233re2).

CHAPTER VI: FUTURE PERSPECTIVES

The works integrating this thesis provided insightful clues into the role of genetic polymorphisms in PGE_2 pathway genes on colorectal carcinogenesis that need to be further explored.

To assure the generasibility of our findings and to better estimate the interaction between locus and the environment larger and replicative studies should be implemented. The control subjects should be screened for colorectal tumors to exclude selection bias. Furthermore, other genes in the metabolism of arachidonic acid, namely the 5 and 12-lipoxigenases (LOX), should be incorporated in the gene-gene interaction analysis considering their potential carcinogenic roles.

An interaction between the rs689466A>G polymorphism and smoking habits was observed in CRC. In the *in vitro* study we observed a higher transcriptional activity of *COX-2* gene in the presence of G allele. Nevertheless, it is important to identify which molecular pathway is involved in this regulation, namely through studies involving EMSA and ChIP methodology. Additionaly, exposing the two CRC cell lines transfected with either allele to carcinogenic components of tabacco smoke might highlight new molecular pathways regulating COX-2 overexpression, and potentially providing new targets for chemoprevention of colorectal cancer.

It would be interesting to increase the number of colorectal tissues in the functional study to assess the influence of gene-gene and gene-environment interactions in the mRNA expression of target genes. Additionally, tissue samples from adenomatous polyps should be included as they might represent a better model when studying polymorphisms involved in the development of early stages colorectal tumors. Moreover, the influence of the genetic variants explored in this thesis, including the gene-gene interactions, on PGE_2 levels should be addressed.

Furthermore, the conclusion of this thesis and further results may be interpreted with costs of procedures and patients' preferences to estimate the cost-benefit of these approaches ie, to alter the screening and follow-up programes according to the genetic background.

APPENDIX: FUNCTIONAL POLYMORPHISMS OF TOLL-LIKE RECEPTORS 2 AND 4 ALTER THE RISK FOR COLORECTAL CARCINOMA IN EUROPEANS.

G Model
YDLD-2238; No. of Pages 7

ARTICLE IN PRESS

Digestive and Liver Disease xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Oncology

Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans

Pedro Pimentel-Nunes[a,b,*,1], Ana Luísa Teixeira[c,d,1], Carina Pereira[c,d], Mónica Gomes[c,d], Catarina Brandão[b], Catarina Rodrigues[e], Nádia Gonçalves[a], Inês Boal-Carvalho[a], Roberto Roncon-Albuquerque Jr.[a], Luís Moreira-Dias[b], Adelino F. Leite-Moreira[a], Rui Medeiros[c,d], Mário Dinis-Ribeiro[b,f]

[a] Department of Physiology and Cardiothoracic Surgery, Cardiovascular Research & Development Unit, Faculty of Medicine, University of Porto, Portugal
[b] Gastroenterology Department, Portuguese Oncology Institute, Porto, Portugal
[c] Molecular Oncology Group, Portuguese Oncology Institute, Porto, Portugal
[d] Abel Salazar Biomedical Sciences Institute, ICBAS, University of Porto, Portugal
[e] Oncology Department, CHTMAD-EPE, Vila Real, Portugal
[f] CINTESIS/Department of Health Information and Decision Sciences, Porto Faculty of Medicine, Porto, Portugal

ARTICLE INFO

Article history:
Received 2 May 2012
Accepted 8 August 2012
Available online xxx

Keywords:
Colorectal cancer
Single nucleotide polymorphisms
TLR2
TLR4

ABSTRACT

Background: Colon carcinogenesis is associated with increased expression levels of Toll-like receptor 2 and Toll-like receptor 4.
Aim: To determine in a Caucasian population the role of Toll-like receptor 2 and Toll-like receptor 4 polymorphisms in colorectal cancer development.
Methods: Hospital based multicentre case control study involving 193 colorectal cancer patients and 278 healthy individuals. DNA samples were extracted from blood cells and genotyping of *TLR2+597T>C*, *TLR2−4760T>C*, *TLR4−3745A>G*, *TLR2Arg753Gln*, *TLR4Asp299Gly* was performed. Functionality of risk polymorphisms was evaluated through production of TNF-α in cell culture and Toll-like receptors levels quantified by real-time RT-PCR.
Results: TLR2+597CC homozygous had 5-fold decreased risk (odds ratio (OR) = 0.21, 95% CI: 0.09–0.50, $p < 0.001$) and *TLR4 299Gly* homozygous 3-fold increased risk of colorectal cancer (OR = 3.30, 95% CI: 1.18–9.28, $p = 0.015$). In stratified analysis, *TLR2+597CC* genotype protective effect was even higher in overweight individuals (OR = 0.17, 95% CI: 0.06–0.53, $p < 0.001$) and in never smokers (OR = 0.11, 95% CI: 0.02–0.51, $p = 0.001$). Also, the increased risk effect for *TLR4 299Gly* homozygous genotype was higher in overweight individuals (OR = 8.67, 95% CI: 1.11–87.85, $p = 0.011$). *TLR2+597T>C* polymorphism conferred 41% less ($p = 0.03$) and *TLR4Asp299Gly* 65% more TNF-α production ($p = 0.02$) with no differences in Toll-like receptors levels.
Conclusion: Functional Toll-like receptor 2 and Toll-like receptor 4 polymorphisms significantly alter the risk to have colorectal cancer. Obesity and smoking may influence the risk for colorectal cancer in individuals presenting these genetic profiles.

1. Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, being the third most common in males and the second one in females. Its incidence rates are rapidly increasing in several areas in the world, probably related to a combination of factors like diet, obesity and smoking [1–3]. It is clear that there are at least three distinct molecular pathways for CRC development [4,5]. Nevertheless, modifier genes and inflammatory molecules, by promoting genomic instability and controlling cell growing, may also be important for the progression of these CRC carcinogenic pathways [6–8]. Indeed, COX-2 polymorphisms have been associated to CRC risk, suggesting that other factors, namely pro-inflammatory ones, significantly influence the adenoma–carcinoma sequence [9–11].

Toll-like receptors (TLR) are key players in immune system, with ten different TLRs being expressed in humans [12,13]. TLR2

* Corresponding author at: Al. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal. Tel.: +351 96 7340096; fax: +351 22 5513646.
E-mail address: pedronunesml@gmail.com (P. Pimentel-Nunes).
[1] The authors equally contributed to this study and should be considered joint first authors.

1590-8658/$36.00 © 2012 Editrice Gastroenterologica Italiana S.r.l. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dld.2012.08.006

recognizes a number of pathogen-associated molecular patterns (PAMP) from Gram positive bacteria and TLR4 is the receptor of the Gram negative bacteria lipopolysaccharide (LPS) [14–16]. Activation of these receptors initiates intracellular signalling pathways that promote cell survival and production of different pro-inflammatory mediators such as COX-2 [12,17–20]. Because they are not only intrinsically related to inflammation but also to cell survival signalling, epithelial regeneration and cell proliferation, recent reports associate these receptors function to tumourigenesis [21,22]. Concerning gastrointestinal system, current evidence suggests that TLR innate immune responses to PAMPs from luminal microbiota may be essential for the development of tumours [21–24]. In fact, our own group and other authors have shown that human colon carcinogenesis is associated with increasing expression levels of TLR2 and TLR4 [25–28].

Playing an important role in the interface between host and the environment, dysregulation of the TLR2 and TLR4 signalling pathways due to functional single nucleotide polymorphisms (SNPs) can disrupt the normal cellular immune response and consequently conditioning cytokines cellular levels, contributing for inflammation and cancer. Genetic variants in TLRs encoding genes may contribute to different response phenotypes, including susceptibility to cancer development.

A potential functional genetic polymorphism in TLR4 gene has been described responsible for an A-to-G transition in exon 3, causing an aspartic acid/glycine substitution Asp299Gly (rs4986790). This transition affects the extracellular domain of TLR4 receptor, in a ligand-recognition area [29]. *TLR4 Asp299Gly* polymorphism has been subject of investigation in several studies involving different types of cancer [30–35]. Despite some studies observed lack association of *TLR4 Asp299Gly* polymorphism and the risk of CRC development [36,37], one study associated this SNP to CRC [38] and others address its role in tumour prognosis [39,40]. Several TLR2 SNPs have also been associated to cancer [38,40,41], namely, it has been reported, that TLR2+597T>C (rs3804099) polymorphism can alter the risk of colon cancer development [40].

We hypothesized genetic SNPs, with potential influence on TLR2 and TLR4 receptor expression and/or function, may have impact in CRC development. Our purpose was to address the role of potential functional TLR SNPs on CRC risk in a European Caucasian population.

2. Materials and methods

2.1. Study population and data collection

The study population has been described previously [11]. This hospital-based case–control study included 471 participants: 193 histologically confirmed CRC patients and 278 cancer-free controls from the northern and central region of Portugal recruited at the Portuguese Institute of Oncology, Porto (IPOP) and Coimbra (IPOC). Eligible cases included patients aged 50–75 years with a newly diagnosed of CRC between January 2002 and September 2007 and CRC patients submitted to chemotherapy between January 2004 and March 2008 that were under follow-up between February and March 2008 at IPOP and IPOC. Controls were healthy individuals aged 50 years or more without clinical evidence of cancer (blood donors) recruited at IPOP between July 2005 and October 2007. The characteristics of the study population are summarized in Table 1. Cases were significantly older than controls' with a median age of 62 years (50–75) [vs 56 years in controls (50–65), $p < 0.001$]. There were no significant differences in the distribution of gender, BMI and smoking habits between both groups. Written informed consent was obtained from all participants before their inclusion in the study, according to the Declaration of Helsinki.

Table 1
Description of participants (cases and controls): age, gender, body mass index, smoking habits, and summarized clinical characteristics of cases (patients with cancer).

	Cases $n = 193$	Controls $n = 278$	p
Demographics			
Age (years)			
Mean (SD)	62 (7)	56 (4)	
Median [min–máx]	62 [50–75]	55 [50–65]	<0.001
Gender, *n* (%)			
Male	123 (64)	176 (63)	0.926
Female	70 (36)	102 (37)	
Lifestyle behaviours[b]			
BMI category[a], *n* (%)			
<25 kg/m^2	49 (26)	41 (23)	0.598
≥25 kg/m^2	143 (74)	136 (77)	
Smoking status, *n* (%)			
Never smokers	142 (74)	110 (66)	0.095
Ever smokers	51 (26)	58 (34)	
Tumour characteristics			
Tumour location, *n* (%)			
Rectum	82 (42)	–	
Colon	111 (58)	–	
Stage, *n* (%)			
I or II	76 (40)	–	
III or IV	116 (60)	–	

BMI, body mass index.
[a] Categorization based on the cut-off defined by WHO for overweight people.
[b] The numbers may not add-up since we were unable to gathered this information for all subjects, namely in controls' group.

Furthermore, the Ethics Committee of the IPOP and IPOC approved this research.

2.2. Sample DNA extraction and TLR2/TLR4 polymorphisms genotyping

Genomic DNA was extracted from peripheral blood leukocytes, using the QIAamp® DNA Blood Mini Kit (Qiagen, Madrid, Spain) following manufacturer's instructions.

The selection of studied TLR2 and TLR4 polymorphisms was based on expected functional repercussion (FastSNP) and/or previous associations with cancer development of SNPs retrieved from literature and public database search (dbSNP). The following polymorphisms were selected: TLR2 Arg753Gln (rs5743708), TLR2−4789T>C (rs4696483), TLR2+597T>C (rs3804099); TLR4 Asp299Gly (rs4986790) and TLR4−3869A>G (rs2737191). TLR2 Arg753Gln and TLR4 Asp299Gly variants were analysed through PCR-RFLP method. Briefly, DNA was amplified in a 50-μL reaction mixture containing TLR4 Asp299Gly primers (forward, 5′-AGC ATA CTT AGA CTA CTA CCT CCA TG-3′; reverse, 5′-GAG AGA TTT GAG TTT CAA TGT GGG-3′), and TLR2 Arg753Gln primers (forward, 5′-CAT TCC CCA GCG CTT CTG CAA GCT CC-3′; reverse, 5′-GGA ACC TAG GAC TTT ATC GCA GCT C-3′) (Metabion Martinsried, Germany), respectively, 1× PCR buffer, 1 unit Taq polymerase, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphates, and 20 ng DNA. TLR2 PCR products (129 bp) were incubated with MspI restriction endonuclease at 37 °C, in the presence of allele G the fragment is cleaved by the enzyme giving arise two fragments (104 and 25 bp), whereas the A allele is not cleaved by the enzyme. TLR4 PCR products (188 bp) were incubated overnight with NcoI restriction endonuclease at 37 °C, the polymorphism was defined by the presence (G) or absence (A) of a restriction site. TLR2+597T>C, TLR2−4789T>C and TLR4−3869A>G polymorphisms were analysed by allelic discrimination using 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR were carried out using a 6-mL reaction mixture, containing 1× Master Mix (Applied Biosystems), with 1× probes (TaqMan assay, C_22274563_10, C_27313261_10, C_1844485_10, respectively

Please cite this article in press as: Pimentel-Nunes P, et al. Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. Dig Liver Dis (2012), http://dx.doi.org/10.1016/j.dld.2012.08.006

Table 2
TLR-2+597T>C and *TLR-4 Asp299Gly* polymorphisms-related odds ratios for colorectal cancer and genotype frequencies in patients and controls.

	Controls *n* (%)	Cases *n* (%)	OR	95% CI	*p*
TLR-2+597T>C					
TT/TC	235 (86)	184 (97)			
CC	37 (14)	6 (3)	**0.21**	**0.086–0.501**	**<0.001**
TLR-4 Asp299Gly					
AA/AG	186 (97)	169 (92)			
GG	5 (3)	15 (8)	**3.30**	**1.175–9.279**	**0.015**

OR, odds ratio; 95% CI, 95% confidence interval. Bold values represent statistical significant results and the significance of that values (*p*) is in the right column.

Applied Biosystems) and 20 ng of the DNA sample. Quality control procedures implemented for genotyping included double sampling in about 10% of the samples to assess reliability and the use of negative controls to step-away false-positives. In PCR-RFLP method, two authors obtained the results independently, and the ambiguous were reanalysed.

2.3. Functional evaluation of TLR's genotypes – culture and activation of peripheral blood monocytes (PBM)

Blood samples were obtained from 14 healthy blood donors according to the different genotype of *TLR2+597T>C* and *TLR4 Asp299Gly* polymorphisms. Our culture cell protocol was described elsewhere [42]. Briefly, PBM were isolated from whole blood by density-gradient centrifugation with Ficoll-Paque (GE Healthcare Lifesciences, UK) followed by positive selection isolation with anti-CD11b Microbeads (MACS, Miltenyi Biotec, Germany). Afterwards, PBM primary culture was performed. The monocytes samples were adjusted to 1×10^5 cells per well and cultured in quadruplicate in RPMI-1640 medium (GE Healthcare Lifesciences, UK), supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine and 12% foetal bovine serum (GE Healthcare Lifesciences, UK) at 37 °C and 5% of CO_2. After 3 h incubation, nonadherent cells and supernatants were removed and fresh medium was added (time 0 h). PBMs from the different genotypes were separately incubated in four different wells with zymosan (Zym) [2 μg/mL] for TLR2/TLR6 stimulation, with Lipopeptide (Lp) Pam3Cys-SK4 [1 μg/mL] for TLR2/TLR1 activation, with LPS [1 μg/mL] for TLR4 stimulation, and 0.9% NaCl as internal control. The supernatants were collected after 24 h stimulation. After collection, supernatants were frozen at −80 °C until analysis of TNF-α levels (R&D Systems, USA; sensitivity 1.6 pg/mL).

2.4. Isolation of mRNA from PBM and quantification of TLR2 of TLR4 expression

These methods were described elsewhere [42]. Briefly, after separation and isolation of PBM, 1×10^5 cells were collected and the final cell pellet was used for mRNA isolation with TriPure Isolation Reagent (Roche, Germany). Two-step real-time RT-PCR was used to perform relative quantification of mRNA. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and PCR threshold cycle of graded dilutions from a randomly selected sample. For relative quantification of specific mRNA levels, 100 ng of total mRNA from each sample underwent two-step real-time RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were similar in all genotypes, which enabled the use of this gene as internal control. Specific PCR primers pairs for the studied genes were: **GAPDH** – fw (P1) 5′-TTG GCC AGG GGT GCT AAG-3′ and rev (P2) 5′-AGC CAA AAG GGT CAT CAT CTC-3′; **TLR2** – fw 5′-GAT CCC AAC TAG ACA AAG ACT-3′ and rev 5′-CTG CGG AAG ATA ATG AAC ACC-3′; **TLR4** – fw 5′-CTA AAC CAG CCA GAC CTT GAA-3′ and rev 5′-ACC TGT CCC TGA ACC CTA TGA-3′. Results of mRNA quantification were expressed as the ratio gene/GAPDH.

2.5. Statistical analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences – SPSS for Windows (version 17.0). The Hardy–Weinberg equilibrium was tested by a Pearson goodness-of-fit test to compare the observed vs the expected genotype frequencies. Chi-square analysis was used to compare categorical variables, using a 5% level of significance. Statistical differences between mean values were evaluated applying the Mann–Whitney test. Multivariate logistic regression analysis was used to estimate odds ratio (OR) and its 95% confidence interval (CI) as a measure of the association between variant allele carriers and the risk for the development of CRC. The potential confounding variables: age, gender, BMI and smoking habits were addressed through data stratification. For each OR estimation dominant and recessive models of analysis were followed and results presented according to the tendency observed. The Kaplan–Meier method and log-rank test were used to compare genotype influence in the age at CRC diagnose. One-way ANOVA and Student's *t* test for paired and unpaired data (or correspondent non-parametric test) were used for group comparison of TNF-α production in cell culture and for mRNA levels. Statistical significance was set at $p < 0.05$.

3. Results

3.1. SNP analysis and risk evaluation

We did not find any differences between cases and controls concerning *TLR2−4760T>C*, *TLR2Arg753Gln* and *TLR4−3745A>G* polymorphisms. *TLR2+597T>C* and TLR4 *Asp299Gly* polymorphisms genotypes' distribution in cases and controls and genetic profile-associated risk of CRC are presented in Table 2. According to *TLR2+597T>C* polymorphism, the CC genotype was under-represented in CRC group (3% vs 14% in controls', $p < 0.001$). The present results show lower risk for developing CRC in CC genotypes carriers than in those individuals' carriers of TT/TC genotypes (OR = 0.21, 95% CI: 0.09–0.50, $p < 0.001$). In *TLR4 Asp299Gly* genotype distribution, we observed that GG genotype was more frequent in CRC group than in control group (8% vs 3%, $p = 0.015$). Furthermore, we observed that GG genotype carriers had higher risk for developing CRC than AA/AG genotype carriers (OR = 3.30, 95% CI: 1.18–9.28, $p = 0.015$). We observed an interaction between *TLR2+597T>C* polymorphism and BMI and smoking status but not with gender (Table 3). Both, female and male CC genotype carriers had lower risk to CRC development (OR = 0.10, 95% CI: 0.01–0.76, $p = 0.005$ and OR = 0.27, 95% CI: 0.10–0.73, $p = 0.004$, respectively). We observed that CC genotype is associated with lower risk to CRC development in individuals with BMI ≥ 25 (OR = 0.17, 95% CI: 0.06–0.53, $p < 0.001$) and in individuals never smokers (OR = 0.11, 95% CI: 0.24–0.51, $p = 0.001$). Concerning the *TLR4 Asp299Gly*

Please cite this article in press as: Pimentel-Nunes P, et al. Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. Dig Liver Dis (2012), http://dx.doi.org/10.1016/j.dld.2012.08.006

Table 3
Potential interaction between gender, body mass index, smoking status and *TLR-2+597T>C and TLR-4 Asp299Gly* polymorphisms in the development of colorectal cancer.

	Controls *n* (%)	Cases *n* (%)	OR	95% CI	*p*
TLR-2+597T>C					
Stratification					
Gender					
Female					
TT/TC	84 (87)	67 (98)			
CC	13 (13)	1 (2)	**0.10**	**0.012–0756**	**0.005**
Male					
TT/TC	151 (86)	117 (96)			
CC	24 (14)	5 (4)	**0.27**	**0.100–0.726**	**0.004**
BMI					
<25					
TT/TC	37 (97)	46 (96)			
CC	1 (3)	2 (4)	1.61	0.140–18.441	0.588
≥25					
TT/TC	114 (86)	138 (97)			
CC	19 (14)	4 (3)	**0.17**	**0.058–0.526**	**0.001**
Smoking status					
Never smokers					
TT/TC	92 (88)	138 (99)			
CC	12 (12)	2 (1)	**0.11**	**0.024–0.508**	**0.001**
Ever smokers					
TT/TC	50 (86)	46 (8)			
CC	8 (14)	4 (8)	0.54	0.153–1.026	0.260
TLR-4 Asp299Gly					
Stratification					
Gender					
Female					
AA/AG	57 (98)	61 (91)			
GG	1 (2)	6 (9)	5.61	0.655–48.015	0.083
Male					
AA/AG	129 (97)	87 (92)			
GG	4 (3)	9 (8)	2.77	0.805–8.969	0.084
BMI					
<25					
AA/AG	27 (96)	42 (93)			
GG	1 (4)	3 (7)	1.93	0.191–19.512	0.502
≥25					
AA/AG	91 (99)	126 (91)			
GG	1 (1)	12 (9)	**8.67**	**1.107–87.845**	**0.011**
Smoking status					
Never smokers					
AA/AG	74 (100)	123 (91)			
GG	1 (1)	12 (9)	**7.22**	**1.08–56.660**	**0.004**
Ever smokers					
AA/AG	36 (95)	46 (94)			
GG	2 (5)	3 (6)	1.174	0.186–7.403	0.620

OR, odds ratio; 95% CI, 95% confidence interval. Bold values represent statistical significant results and the significance of that values (*p*) is in the right column.

polymorphism, we observed that individual' carriers of GG genotype and BMI ≥ 25 had a higher risk to CRC development (OR = 8.67, 95% CI: 1.11–87.85, $p = 0.011$). Furthermore, the GG genotype was also associated with risk to CRC development in never smokers' individuals (OR = 7.22, 95% CI: 1.08–56.67, $p = 0.004$). No difference was found considering different cancer locations, namely comparing rectal or colon cancer.

3.2. Influence of TLR2+597T>C and TLR4 Asp299Gly polymorphisms on the time-to-diagnosis of CRC

When we evaluated the influence of *TLR2+597T>C* polymorphism in the age at CRC diagnose (Fig. 1), we observed that the TT/TC genotype carriers tend to be younger than CC genotype carriers at diagnose (66 vs 69 years, $p = 0.073$, respectively). Concerning *TLR4 Asp299Gly* polymorphism we observed a lack of association of the polymorphism and the age at CRC, despite GG carriers being younger at the age of diagnosis (GG vs AA/AG, 63 vs 65 years, $p = 0.4$ respectively). No other statistical important association or tendency between the studied polymorphisms and any other clinical parameter (e.g. survival, answer to therapy) was found.

3.3. Functional characterization of TLR2+597T>C and TLR4 Asp299Gly polymorphisms

The genotypes of the 14 participants involved in the functional study were: *TLR2+597T>C*, 5 CC, 6 TC and 3 TT; *TLR4Asp299Gly*, 5 GG, 2 AG and 7 AA. Statistical differences were found when comparing *TLR2+597T>C* CC homozygous with T carriers after Lp stimulation (TNF-α production of 127.0 ± 18.7 vs 214.3 ± 23.2 pg/mL, $p = 0.03$) and after LPS stimulation when comparing *TLR4 299Gly* carriers vs AA homozygous (TNF-α production of 259.5 ± 27.7 vs 157.9 ± 22.2 pg/mL, $p = 0.02$) (Fig. 2). The TLR2 mRNA levels for the different TLR2 genotypes were 0.58 ± 0.11 (CC), 0.41 ± 0.08 (CT) and 0.47 ± 0.17 (TT) and the TLR4 mRNA levels for the different TLR4 genotypes were 1.5 ± 0.59 (AA), 0.52 ± 0.35 (AG) and 0.87 ± 0.26 (GG), without any statistical difference between the groups. T carriers for *TLR2+597T>C* had TLR2 levels of 0.42 ± 0.07 ($p = 0.2$ vs CC

Please cite this article in press as: Pimentel-Nunes P, et al. Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. Dig Liver Dis (2012), http://dx.doi.org/10.1016/j.dld.2012.08.006

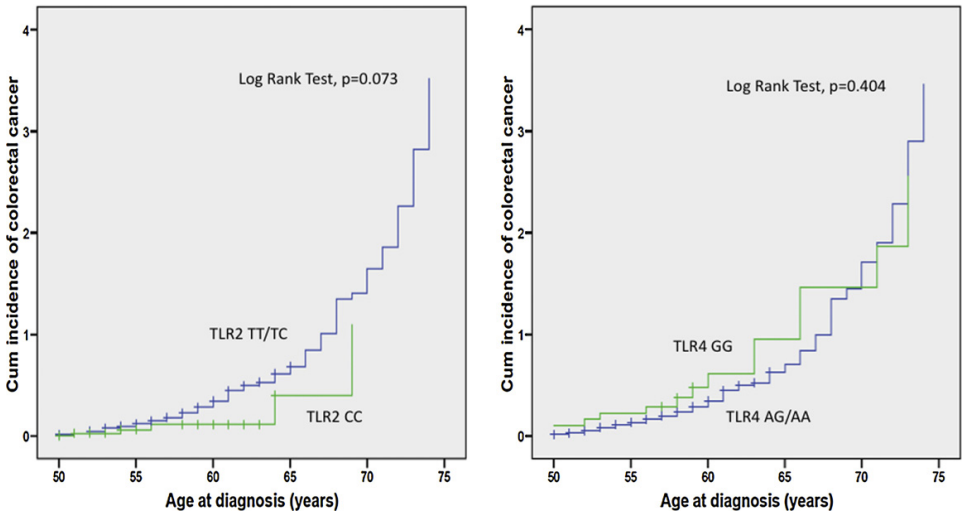


Fig. 1. TLR2 (*TLR-2+597T>C*) and TLR4 (*TLR-4 Asp299Gly*) genotype influence in the age of CRC diagnosis (Kaplan–Meier curves and log-rank test). The effect of the *TLR-2+597T>C* CC genotype in the age of diagnosis was stronger than the *TLR4 299Gly* homozygous genotype.

homozygous) and G carriers of *TLR4Asp299Gly* had TLR4 levels of 0.82 ± 0.25 ($p = 0.2$ vs AA homozygous).

4. Discussion

In the present study we describe that functional TLR2 and TLR4 SNPs significantly influence the risk of CRC. Our results suggest that small changes in the normal function of these receptors due to functional SNPs may contribute to an unbalanced cytokine and pro-oncogenic cellular microenvironment and thus to a higher risk for cancer development.

Why should TLRs SNPs influence the risk of CRC development? It is current knowledge that a strict regulation of TLRs activation is fundamental for maintaining colon homeostasis [24]. Normal colon mucosa constitutively express TLRs, however, it also presents a high expression of TLRs inhibitors, like TOLLIP and PPARγ, which circumscribe TLRs protein expression to basolateral membrane where they are not continuously exposed to PAMPs preventing in this way inadequate inflammation to commensal bacteria [43–48]. Basal TLRs expression in that particular cellular location appears important for maintaining gastrointestinal homeostasis, preventing at the same time auto-immunity processes, controlling bacterial infections and allowing epithelial regeneration [21,49–52]. Since TLRs also activate cell survival signalling pathways, abnormal TLR activation could promote colon carcinogenesis [21,22]. Indeed, several groups including our own have shown in human studies that colon carcinogenesis is associated with decrease expression of TLRs inhibitors and conversely with higher protein expression of TLR2 and TLR4 [26,28]. It was previously shown that TLR4 expression in tumours may have prognostic value [25,27] and several animal studies suggest that TLR2 and TLR4 activation may be essential for CRC development [23,53–55]. So, it appears that dysregulation of these receptors activation may influence the risk of cancer.

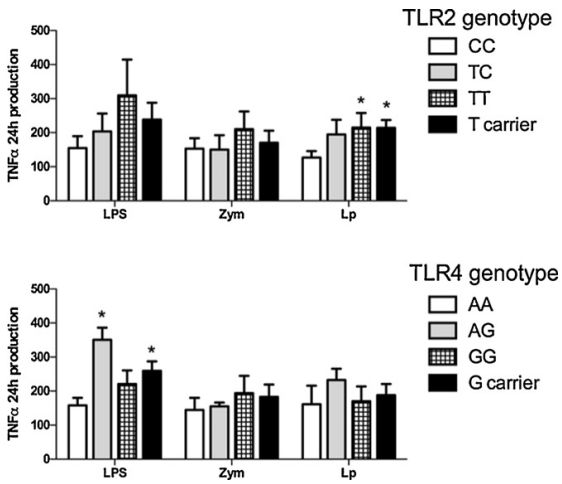


Fig. 2. TNF-α 24 h production (pg/mL) after stimulation with LPS (lipopolysaccharide), Zym (zymosan), and Lp (lipopeptide) in culture cell of monocytes with the different TLR2 (*TLR-2+597T>C*) and TLR4 (*TLR-4 Asp299Gly*) genotypes. *$p < 0.05$ vs CC (TLR2) or vs AA (TLR4).

In this line of thoughts, we found that *TLR2+597T>C* and *TLR4 Asp299Gly* SNPs significantly influence the risk of CRC development, suggesting that these TLRs SNPs may be genetic susceptibility markers for CRC. The CC genotype of the *TLR2+597T>C* SNP was associated with 5-fold decreased risk of CRC development (OR = 0.21), which is a remarkable result for a SNP. In our study, the CC genotype frequency in controls was similar to that observed in healthy European Caucasian [56] and Korean individuals [57] and higher than that observed in Thailand [58]. The *TLR2+597T>C* polymorphism in exon 3 does not appear to induce any amino acid change, remaining its functional impact and molecular mechanism poor understood. According to in silico analysis, this SNP can introduce alterations in splicing regulation, possibly leading to an alteration in TLR2 molecule. On the other hand, it may be in linkage disequilibrium with another functional SNP in TLR2 and thereby influencing promoter activity or the stability of the transcript [59]. Previous reports have shown associations of this polymorphism in TLR2 gene with melanoma susceptibility [41], sepsis [60] and reverse reaction in leprosy [59]. To the best of our knowledge we showed for the first time that TLR2+597T>C SNP may confer hypofunctionality to the receptor. Indeed, monocytes with the CC genotype produced 41% less TNF-α in cell culture. Moreover, we did not find any differences in TLR2 levels between the different

Please cite this article in press as: Pimentel-Nunes P, et al. Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. Dig Liver Dis (2012), http://dx.doi.org/10.1016/j.dld.2012.08.006

genotypes suggesting that the potential hypofunctionality conferred by this polymorphism is not dependent of TLR2 levels. So, for any given stimulus, individuals with this SNP may have less production of inflammatory cytokines and less cell survival signalling and this might help to explain the increase risk of melanoma and sepsis and the decrease risk of CRC with an early age of diagnosis.

The *TLR4 Asp299Gly* polymorphism leads to missense replacement of a conserved aspartic acid residue with a glycine amino acid that alters the structure of the extracellular domain of this receptor. *TLR4 Asp299Gly* has been subject of investigation in several studies involving different type of cancer with controversial results [31,33,34,39,61–64]. Due to evolutionary pressure and human migration, this TLR4 polymorphism has a distinct distribution in different populations, and may or not be cosegregated with the *TLR4 Thr399Ile* polymorphism, which may change the functionality of the receptor and may help to explain the discrepancies between the studies [65]. We observed that in our control population the frequency of *TLR4 Asp299Gly* polymorphism tend to be similar to those observed in healthy European Caucasian [31,33,34] and American [61,62] populations. The significance of this SNP led to contradictory conclusions about its functional role [66]. Studies performed by Arbour et al. reported that this SNP was associated with a blunted response o inhaled LPS [29]. However, Lundberg et al. suggested that not only genetic variant in TLR4 should be considered in functional studies but also the origin of LPS [67]. Furthermore, Ferwerda et al. showed that cells from individuals' carriers of *299Gly* variant significantly produce higher amounts of pro-inflammatory cytokines than homozygous wild-type [65]. Recently, a study performed by Eyking et al. demonstrated that Caco-2 cells which expressed *TLR4 Asp29Gly* polymorphism had a significant increase in expression levels of genes associated with inflammation and/or tumourigenesis compared with cells that expressed other forms of TLR4 [68]. Our results, although they are not definitive concerning the functionality of this SNP, are in agreement with the results of Eyking et al. showing that monocytes from carriers of G allele produce 64% more TNF-α when stimulated with LPS. Clinical studies also confirm the oncogenic potential of this SNPs since this variant allele has been associated with a more quickly relapse in patients submitted to radiotherapy and chemotherapy [30]. Other study showed that this TLR4 SNP might alter prognosis on patients that receive oxaliplatin [37]. So, this gain-of-function genetic variant implies the *TLR4 Asp299Gly* in malignant progression of human colon cancer [68]. Future studies should study the role of these SNPs also for prognosis and answer to therapy.

The other aspect that is interesting in our study is that both the protective effect of the *TLR2+597T>C* SNP and the risk effect of the *TLR4 Asp299Gly* SNP appear to be stronger in overweight and never smokers' individuals. Obesity is a well known risk factor for CRC and in last decade, increase evidence has suggested the relevance of a chronic inflammatory state in obesity [69]. Long-term smoking also causes systemic inflammation with an increase of inflammatory mediators concentration (C-reactive protein, IL-6, IL-8, TNFα) [70]. Indeed, and in agreement with our results, recently it was shown that *TLR2+597T>C* polymorphism can interact with nonsteroidal anti-inflammatory drug use and cigarette smoking to alter risk of colon cancer [40]. Individuals never-smokers and CC genotype carriers have even lower risk for CRC development probably due to their genetic background, with attenuated TLR2 function, and due to lower exposure to environmental factors. In that line of thoughts it is easily understood why overweight individuals *TLR4 Asp299Gly* homozygous have greater risk of cancer, however, why never smokers have greater risk than smokers is not so comprehensive. We may speculate that the genetic influence of the TLR4 Asp299Gly SNP may be blunted in the face of the deleterious effect of smoking and, so, this SNP may strongly interact with the inflammatory process of obesity but not with the distinctive inflammation process of smoking. Indeed, it is well known that TLR4 may have an important influence on adiposity and metabolic syndrome [71].

Two main drawbacks could be noticeable in our study. First, even if a match for ages were attempted by including only controls aged 50 or more, a difference of ages between cases and controls existed and secondly, the level of certainty of absence of CRC among controls. The first was addressed in the statistical analysis and for the second point, we should consider that controls were recruited in 2005–2007, and, up to now, 85% of them (235/278) were asymptomatic and still blood donors, so no clinical evidence of CRC is present 5 years after the recruitment. Moreover, of the 43 controls that were not blood donors in 2012, 31 quit because of age criteria and there were no record of CRC in any of the 278 participants 5–7 years after recruitment. Thus, taking altogether, we may well consider that our control population represents individuals without CRC and that the difference of ages at the time of recruitment was not an issue to our results and conclusions.

In conclusion, functional TLRs SNPs modulate in a significant way the individual susceptibility for CRC development with the *TLR2+597CC* genotype decreasing 5-fold, whereas *TLR4 299Gly* homozygous genotype increasing 3-fold the CRC risk. Factors like obesity and smoking habits may influence the risk of CRC in individuals presenting these genetic profiles. In future, the identification of these genetic profiles may help to define more efficacious strategies for screening of CRC through an individual fitted schedule.

Conflict of interest statement

None to declare.

Acknowledgments

This study was supported by grants for medical investigation from Portuguese Oncology Institute of Porto. ALT is a recipients of a Doctoral degree grant from FCT (SFRH/BD/47381/2008).

The authors are sincerely grateful to Paulo Torres and Luísa Lopes dos Santos for all the collaboration concerning the blood donors.

None of the authors have any disclosure.

The results of this article were partially presented as an oral communication in 18th UEGW 2010 Barcelona.

References

[1] Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA: A Cancer Journal for Clinicians 2011;61:69–90.
[2] Jemal A, Siegel R, Xu J, et al. Cancer statistics, 2010. CA: A Cancer Journal for Clinicians 2010;60:277–300.
[3] Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. CA: A Cancer Journal for Clinicians 2005;55:74–108.
[4] Ahnen DJ. The American College of Gastroenterology Emily Couric Lecture—the adenoma–carcinoma sequence revisited: has the era of genetic tailoring finally arrived? The American Journal of Gastroenterology 2011;106:190–8.
[5] Noffsinger AE. Serrated polyps and colorectal cancer: new pathway to malignancy. Annual Review of Pathology 2009;4:343–64.
[6] Eberhart CE, Coffey RJ, Radhika A, et al. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107:1183–8.
[7] Sarraf P, Mueller E, Smith WM, et al. Loss-of-function mutations in PPAR gamma associated with human colon cancer. Molecular Cell 1999;3:799–804.
[8] Sheng H, Shao J, Kirkland SC, et al. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. Journal of Clinical Investigation 1997;99:2254–9.
[9] Hoff JH, te Morsche RH, Roelofs HM, et al. COX-2 polymorphisms -765G->C and -1195A->G and colorectal cancer risk. World Journal of Gastroenterology 2009;15:4561–5.
[10] Pereira C, Medeiros RM, Dinis-Ribeiro MJ. Cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis: are conclusive results available? European Journal of Gastroenterology and Hepatology 2009;21:76–91.
[11] Pereira C, Pimentel-Nunes P, Brandao C, et al. COX-2 polymorphisms and colorectal cancer risk: a strategy for chemoprevention. European Journal of Gastroenterology and Hepatology 2010;22:607–13.

Please cite this article in press as: Pimentel-Nunes P, et al. Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. Dig Liver Dis (2012), http://dx.doi.org/10.1016/j.dld.2012.08.006

[12] Akira S, Takeda K. Toll-like receptor signalling. Nature Reviews Immunology 2004;4:499–511.
[13] Kawai T, Akira S. TLR signaling. Cell Death and Differentiation 2006;13:816–25.
[14] Akira S, Hemmi H. Recognition of pathogen-associated molecular patterns by TLR family. Immunology Letters 2003;85:85–95.
[15] Takeda K, Kaisho T, Akira S. Toll-like receptors. Annual Review of Immunology 2003;21:335–76.
[16] Kaisho T, Akira S. Pleiotropic function of Toll-like receptors. Microbes and Infection 2004;6:1388–94.
[17] Fukata M, Chen A, Klepper A, et al. Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: role in proliferation and apoptosis in the intestine. Gastroenterology 2006;131:862–77.
[18] Chang YJ, Wu MS, Lin JT, et al. Helicobacter pylori-induced invasion and angiogenesis of gastric cells is mediated by cyclooxygenase-2 induction through TLR2/TLR9 and promoter regulation. Journal of Immunology 2005;175:8242–52.
[19] Spitzer JA, Zheng M, Kolls JK, et al. Ethanol and LPS modulate NF-kappaB activation, inducible NO synthase and COX-2 gene expression in rat liver cells in vivo. Frontiers in Bioscience 2002;7:a99–108.
[20] Lee IT, Lee CW, Tung WH, et al. Cooperation of TLR2 with MyD88, PI3K, and Rac1 in lipoteichoic acid-induced cPLA2/COX-2-dependent airway inflammatory responses. American Journal of Pathology 2010;176:1671–84.
[21] Fukata M, Abreu MT. Role of Toll-like receptors in gastrointestinal malignancies. Oncogene 2008;27:234–43.
[22] Fukata M, Abreu MT. Pathogen recognition receptors, cancer and inflammation in the gut. Current Opinion in Pharmacology 2009;9:680–7.
[23] Fukata M, Chen A, Vamadevan AS, et al. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. Gastroenterology 2007;133:1869–81.
[24] Pimentel-Nunes P, Soares JB, Roncon-Albuquerque Jr R, et al. Toll-like receptors as therapeutic targets in gastrointestinal diseases. Expert Opinion on Therapeutic Targets 2010;14:347–68.
[25] Cammarota R, Bertolini V, Pennesi G, et al. The tumor microenvironment of colorectal cancer: stromal TLR-4 expression as a potential prognostic marker. Journal of Translational Medicine 2010;8:112.
[26] Nihon-Yanagi Y, Terai K, Murano T, et al. Tissue expression of Toll-like receptors 2 and 4 in sporadic human colorectal cancer. Cancer Immunology, Immunotherapy 2012;61:71–7.
[27] Wang EL, Qian ZR, Nakasono M, et al. High expression of Toll-like receptor 4/myeloid differentiation factor 88 signals correlates with poor prognosis in colorectal cancer. British Journal of Cancer 2010;102:908–15.
[28] Pimentel-Nunes P, Goncalves N, Boal-Carvalho I, et al. Decreased Toll-interacting protein and peroxisome proliferator-activated receptor gamma are associated with increased expression of Toll-like receptors in colon carcinogenesis. Journal of Clinical Pathology 2012;65:302–8.
[29] Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nature Genetics 2000;25:187–91.
[30] Apetoh L, Ghiringhelli F, Tesniere A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nature Medicine 2007;13:1050–9.
[31] Garza-Gonzalez E, Bosques-Padilla FJ, Mendoza-Ibarra SI, et al. Assessment of the toll-like receptor 4 Asp299Gly, Thr399Ile and interleukin-8-251 polymorphisms in the risk for the development of distal gastric cancer. BMC Cancer 2007;7:70.
[32] Guo Q, Zhu J, Xia B. Polymorphism of CD14 gene but not the mutation of TLR4 gene is associated with colorectal cancer in Chinese patients. Journal of Gastroenterology and Hepatology 2006;21:92–7.
[33] Landi S, Gemignani F, Bottari F, et al. Polymorphisms within inflammatory genes and colorectal cancer. Journal of Negative Results in Biomedicine 2006;5:15.
[34] Santini D, Angeletti S, Ruzzo A, et al. Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in gastric cancer of intestinal and diffuse histotypes. Clinical and Experimental Immunology 2008;154:360–4.
[35] Zheng SL, Augustsson-Balter K, Chang B, et al. Sequence variants of toll-like receptor 4 are associated with prostate cancer risk: results from the CAncer Prostate in Sweden Study. Cancer Research 2004;64:2918–22.
[36] Davoodi H, Seow HF. Variant Toll-like receptor 4 (Asp299Gly and Thr399Ile alleles) and Toll-like receptor 2 (Arg753Gln and Arg677Trp alleles) in colorectal cancer. Iranian Journal of Allergy, Asthma and Immunology 2011;10:91–9.
[37] Tesniere A, Schlemmer F, Boige V, et al. Immunogenic death of colon cancer cells treated with oxaliplatin. Oncogene 2010;29:482–91.
[38] Boraska Jelavic T, Barisic M, Drmic Hofman I, et al. Microsatelite GT polymorphism in the toll-like receptor 2 is associated with colorectal cancer. Clinical Genetics 2006;70:156–60.
[39] Bergmann C, Bachmann HS, Bankfalvi A, et al. Toll-like receptor 4 single-nucleotide polymorphisms Asp299Gly and Thr399Ile in head and neck squamous cell carcinomas. Journal of Translational Medicine 2011;9:139.
[40] Slattery ML, Herrick JS, Bondurant KL, et al. Toll-like receptor genes and their association with colon and rectal cancer development and prognosis. International Journal of Cancer 2012;130:2974–80, http://dx.doi.org/10.1002/ijc.26314.
[41] Gast A, Bermejo JL, Claus R, et al. Association of inherited variation in Toll-like receptor genes with malignant melanoma susceptibility and survival. PLoS ONE 2011;6:e24370.
[42] Pimentel-Nunes P, Roncon-Albuquerque Jr R, Goncalves N, et al. Attenuation of toll-like receptor 2-mediated innate immune response in patients with alcoholic chronic liver disease. Liver International: Official Journal of the International Association for the Study of the Liver 2010;30:1003–11.
[43] Hopkins PA, Sriskandan S. Mammalian Toll-like receptors: to immunity and beyond. Clinical and Experimental Immunology 2005;140:395–407.
[44] Liew FY, Xu D, Brint EK, et al. Negative regulation of toll-like receptor-mediated immune responses. Nature Reviews Immunology 2005;5:446–58.
[45] Ortega-Cava CF, Ishihara S, Rumi MA, et al. Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. Journal of Immunology 2003;170:3977–85.
[46] Abreu MT, Thomas LS, Arnold ET, et al. TLR signaling at the intestinal epithelial interface. Journal of Endotoxin Research 2003;9:322–30.
[47] Abreu MT, Vora P, Faure E, et al. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. Journal of Immunology 2001;167:1609–16.
[48] Furrie E, Macfarlane S, Thomson G, et al. Toll-like receptors-2, -3 and -4 expression patterns on human colon and their regulation by mucosal-associated bacteria. Immunology 2005;115:565–74.
[49] Beutler BA. TLRs and innate immunity. Blood 2009;113:1399–407.
[50] Fischer M, Ehlers M. Toll-like receptors in autoimmunity. Annals of the New York Academy of Sciences 2008;1143:21–34.
[51] Kluwe J, Mencin A, Schwabe RF. Toll-like receptors, wound healing, and carcinogenesis. Journal of Molecular Medicine 2009;87:125–38.
[52] Pasare C, Medzhitov R. Control of B-cell responses by Toll-like receptors. Nature 2005;438:364–8.
[53] Earl TM, Nicoud IB, Pierce JM, et al. Silencing of TLR4 decreases liver tumor burden in a murine model of colorectal metastasis and hepatic steatosis. Annals of Surgical Oncology 2009;16:1043–50.
[54] Huang B, Zhao J, Li H, et al. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. Cancer Research 2005;65:5009–14.
[55] Yoshioka T, Morimoto Y, Iwagaki H, et al. Bacterial lipopolysaccharide induces transforming growth factor beta and hepatocyte growth factor through toll-like receptor 2 in cultured human colon cancer cells. Journal of International Medical Research 2001;29:409–20.
[56] Jaen O, Petit-Teixeira E, Kirsten H, et al. No evidence of major effects in several Toll-like receptor gene polymorphisms in rheumatoid arthritis. Arthritis Research and Therapy 2009;11:R5.
[57] Kang I, Oh YK, Lee SH, et al. Identification of polymorphisms in the Toll-like receptor gene and the association with allergic rhinitis. European Archives of Oto-Rhino-Laryngology 2010;267:385–9.
[58] Junpee A, Tencomnao T, Sanprasert V, et al. Association between Toll-like receptor 2 (TLR2) polymorphisms and asymptomatic bancroftian filariasis. Parasitology Research 2010;107:807–16.
[59] Bochud PY, Hawn TR, Siddiqui MR, et al. Toll-like receptor 2 (TLR2) polymorphisms are associated with reversal reaction in leprosy. Journal of Infectious Diseases 2008;197:253–61.
[60] Abu-Maziad A, Schaa K, Bell EF, et al. Role of polymorphic variants as genetic modulators of infection in neonatal sepsis. Pediatric Research 2010;68:323–9.
[61] Chen YC, Giovannucci E, Lazarus R, et al. Sequence variants of Toll-like receptor 4 and susceptibility to prostate cancer. Cancer Research 2005;65:11771–8.
[62] Cheng I, Plummer SJ, Casey G, et al. Toll-like receptor 4 genetic variation and advanced prostate cancer risk. Cancer Epidemiology, Biomarkers and Prevention 2007;16:352–5.
[63] Pandey S, Mittal RD, Srivastava M, et al. Impact of Toll-like receptors [TLR] 2 (−196 to −174 del) and TLR 4 (Asp299Gly, Thr399Ile) in cervical cancer susceptibility in North Indian women. Gynecologic Oncology 2009;114:501–5.
[64] Srivastava K, Srivastava A, Kumar A, et al. Significant association between toll-like receptor gene polymorphisms and gallbladder cancer. Liver International: Official Journal of the International Association for the Study of the Liver 2010;30:1067–72.
[65] Ferwerda B, McCall MB, Alonso S, et al. TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. Proceedings of the National Academy of Sciences of the United States of America 2007;104:16645–50.
[66] Ferwerda B, McCall MB, Verheijen K, et al. Functional consequences of toll-like receptor 4 polymorphisms. Molecular Medicine 2008;14:346–52.
[67] Lundberg A, Wikberg LA, Ilonen J, et al. Lipopolysaccharide-induced immune responses in relation to the TLR4 (Asp299Gly) gene polymorphism. Clinical and Vaccine Immunology 2008;15:1878–83.
[68] Eyking A, Ey B, Runzi M, et al. Toll-like receptor 4 variant D299G induces features of neoplastic progression in Caco-2 intestinal cells and is associated with advanced human colon cancer. Gastroenterology 2011;141:2154–65.
[69] Jung C, Gerdes N, Fritzenwanger M, et al. Circulating levels of interleukin-1 family cytokines in overweight adolescents. Mediators of Inflammation 2010;2010:958403.
[70] Tanni SE, Pelegrino NR, Angeleli AY, et al. Smoking status and tumor necrosis factor-alpha mediated systemic inflammation in COPD patients. Journal of Inflammation (London) 2010;7:29.
[71] Pahwa P, Karunanayake CP, Rennie DC, et al. Association of the TLR4 Asp299Gly polymorphism with lung function in relation to body mass index. BMC Pulmonary Medicine 2009;9:46.

Please cite this article in press as: Pimentel-Nunes P, et al. Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. Dig Liver Dis (2012), http://dx.doi.org/10.1016/j.dld.2012.08.006